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(Thermodynamics) "is such a marvelous tool: you don't understand what you put in, and you don't understand what you get out. But you know it must be right."

Aharon Katchalsky
as quoted
by G. Oster (1984)
Nature *309*, 95

Physikalisch-chemische Aspekte immunologischer und anderer reversibler Assoziations-Reaktionen[1])

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Herrn Professor Dr. Dr. I. Trautschold, dem Organisator und Moderator dieser Konferenz, zum ehrenden Andenken

Zusammenfassung: Es werden die grundlegenden Begriffe, Symbole und Formeln reversibler Assoziationsreaktionen am Beispiel von Immunreaktionen zusammenfassend und im Hinblick auf ihre Anwendung im Bereich der Analytik dargestellt (Kapitel 1). Die Enthalpien und Entropien der Aktivierung und Reaktion werden unter Berücksichtigung der Ergebnisse neuerer Untersuchungen gedeutet (Kapitel 2). Die gängigen, auch in den üblichen klinisch-chemischen Laboratorien durchführbaren Verfahren zur Ermittlung physikalisch-chemischer Kenngrößen auch komplexer Assoziationsreaktionen werden diskutiert (Kapitel 3); zur Auswertung der Meßdaten wird auf die kürzlich in dieser Zeitschrift erschienene Übersicht verwiesen (*E. Kuss*, J. Clin. Chem. Clin. Biochem. *20*, 227–234 (1982)). Die Anwendung physikalisch-chemischer Kenngrößen in der analytischen Praxis wird an Beispielen aufgezeigt (Kapitel 4).

Physical chemical aspects of immunological reactions and other reversible associations

Summary: The standard concepts, symbols, and formulae of reversible association reactions are comprehensively illustrated with the aid of the immune reaction, and presented in the context of their use in analysis (section 1). The enthalpy and entropy values of activation and reaction are interpreted in the light of the results of recent investigations (section 2). Common methods that can be performed in the ordinary clinical chemical laboratory are discussed for the determination of physical chemical constants, including those of complicated association reactions (section 3); Processing of experimental data was recently reviewed in this journal (*E. Kuss*, J. Clin. Chem. Clin. Biochem. *20*, 227–234 (1982)). The use of physical chemical constants in analysis is illustrated with examples (section 4).

1. Definitionen

Als immunologische Reaktionen werden die reversiblen Assoziationsreaktionen bezeichnet, die zwischen hydratisierten Immunglobulinen und ihren ebenfalls hydratisierten Antigenen (oder deren spezifischen Kompetitoren) ablaufen, und die zu Immunkomplexen führen. Die bipolaren Zentren der Reaktionen sind in den Bindungsplätzen Q_j der variablen Domäne des Immunglobulins, den möglicherweise unscharf begrenzten Regionen vom Ausmaß etwa 1 nm^2, lokalisiert und in den Bindungsplätzen P_i, den Immundeterminanten oder Epitopen des Antigens, den von etwa 5 Aminosäuren oder Hexosen oder von etwa einem Steroidmolekül ausgefüllten Raumstrukturen. Der endgültige Abstand der sterisch und funktional komplementären Molekülgruppen der beiden Reaktionszentren im Kom-

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

plex wird auf einige 0,1 nm geschätzt. Sterische und funktionale Komplementarität bedingen sich gegenseitig, da die molekularen attraktiven Kräfte nur über kurze Distanzen wirken (1–8).

Die physikalisch chemischen Aspekte der Immunreaktion werden auf die Analytik ausgerichtet, die der Charakterisierung der zwei Gruppen von „Reaktanden", „Bindungsklassen" oder „Bindungsordnungen"

P_i mit den „Ordnungen" i = 1, 2, ... m;
Q_j mit den „Ordnungen" j = 1, 2, ... n;

und den daraus in reversiblen Reaktionen entstehenden oder entstandenen Komplexen, Produkten, P_iQ_j dient:

(Formel 1) $P_i + Q_j \underset{k_{-1ij}}{\overset{k_{+1ij}}{\rightleftarrows}} P_iQ_j$.

„Charakterisierung" beinhaltet die Bestimmung von Qualitäten und Quantitäten der Reaktanden und Produkte. Der Zusammenhang zwischen Qualitäten und Quantitäten zeigt sich im Massenwirkungsgesetz (Gl. 1) und in den damit zusammenhängenden reaktionskinetischen und thermodynamischen Gleichungen. Die möglichen Reaktionspartner P_i, Q_j und ihre Produkte P_iQ_j sind in der Reaktionsmatrix (Abb. 1) aufgeführt. Aus den Spalten und Zeilen der Matrix ergeben sich die Definitionen von p_i und q_j; die Definitionsgleichungen entsprechen dem Massenerhaltungsgesetz (Gl. 2).

(Gl. 1) $$\frac{[P_iQ_j]}{[P_i]\,[Q_j]} = K_{ij} = \frac{k_{+1ij}}{k_{-1ij}} = e^{-\frac{\Delta G^\circ_{ij}}{RT}} = e^{-\frac{\Delta H^\circ_{ij}}{RT}} e^{\frac{\Delta S^\circ_{ij}}{R}}$$

(Gl. 2a) $$p_i = [P_i] + [P_iQ_j] = [P_i]\left(1 + \sum_{j=1}^{n} \frac{K_{ij}\,q_j}{\sum_{a=1}^{m} K_{aj}\,[P_a]}\right)$$

(Gl. 2b) $$q_j = [Q_j] + [P_iQ_j] = [Q_j]\left(1 + \sum_{i=1}^{m} \frac{K_{ij}\,p_i}{\sum_{a=1}^{n} K_{ai}\,[Q_a]}\right)$$

[ ]: Aktivitäten im Reaktionsgleichgewicht (= Konzentrationen mit Aktivitätsfaktor etwa 1)

K_{ij}: Gleichgewichtskonstante der reversiblen Assoziationsreaktion $P_i + Q_j \rightleftarrows P_iQ_j$

k_{+1ij}: Geschwindigkeitskonstante der Hinreaktion $P_i + Q_j \rightarrow P_iQ_j$

k_{-1ij}: Geschwindigkeitskonstante der Rückreaktion $P_iQ_j \rightarrow P_i + Q_j$

R: Gaskonstante = 8,314 J mol^{-1} K^{-1}
= molare *Boltzmann*-Konstante $N\bar{k}$,
N = *Avogadro*-Zahl = 6×10^{23} mol^{-1};
$\bar{k}$ = *Boltzmann*-Konstante
= $1{,}4 \times 10^{-23}$ J K^{-1}

T: Thermodynamische Temperatur ($T_{Eispunkt}$ = 273,15 K)

e: $\lim_{n\to\infty} (1 + 1/n)^n = 2{,}718$
($y = e^x$, dann $\ln y = x$)

ΔG°_{ij}: Standard-Änderung der *Gibbs*-Energie

ΔH°_{ij}: Standard-Änderung der Enthalpie

ΔS°_{ij}: Standard-Änderung der Entropie

i: 1, 2, . . . m

j: 1, 2, . . . n

	P_1	P_2	...	...	P_m	
Q_1	P_1Q_1	P_2Q_1	...	...	P_mQ_1	$Q_1 + \sum_{i=1}^{m} P_iQ_1 = q_1$
Q_2	P_1Q_2	P_2Q_2	...	...	P_mQ_2	$Q_2 + \sum_{i=1}^{m} P_iQ_2 = q_2$
⋮	...	...	...	...	...	.....
⋮	...	...	...	...	...	.....
Q_n	P_1Q_n	P_2Q_n	...	...	P_mQ_n	$Q_n + \sum_{i=1}^{m} P_iQ_n = q_n$
	$P_1 + \sum_{j=1}^{n} P_1Q_j = p_1$	$P_2 + \sum_{j=1}^{n} P_2Q_j = p_2$	...	...	$P_m + \sum_{j=1}^{n} P_mQ_j = p_m$	

Abb. 1. Reaktionsmatrix.

In den Reaktionsansätzen, die als thermodynamische Systeme mit konstantem Druck und konstanter Temperatur betrachtet werden, können P_i und/oder Q_j hinsichtlich ihrer Mengen und Affinitäten *regelmäßig* oder *unregelmäßig* verteilt sein. Als regelmäßig wird eine Verteilung bezeichnet, wenn sich die Mengen symmetrisch um eine Menge mittlerer Affinität verteilen.

Beispiel für regelmäßige Verteilung:

a) monoklonale Antikörper; identische Molekülspezies Q_j mit Anti P_i-Aktivität

b) polyklonale Antikörper mit *Gauss*- oder *Sips*-Verteilung der n verschiedenen Affinitäten der verschiedenen Molekülspezies Q_j mit Anti P_i-Aktivitäten (9).

Beispiel für unregelmäßige multimodale Verteilung:

n diskrete Antikörper-Ordnungen Q_j mit Anti-P_i-Aktivitäten (10).

Der Begriff **Affinität** bleibt thermodynamisch definierten Systemen vorbehalten. Die (relative) Bindungsstärke von Antiseren als Resultante verschiedener, zum Teil gegenläufiger Effekte wird als **Avidität** bezeichnet.

In P_i und/oder Q_j können Ordnungen enthalten sein, deren Elemente zu Aggregaten, z.B. der Art ^nQ (oder ^mP) zusammengeschlossen sind. Von diesen Aggregaten können sequentiell bis zu maximal n P (bzw. bis zu maximal m Q) gebunden werden; in diesen Fällen bezeichnen mp und nq die Konzentrationen der Bindungsplätze und p sowie q die Konzentrationen der Aggregate.

Beispiel für Aggregate:

m = 4: Ribonuclease (M_r = 14000) mit 4 verschiedenen Bindungsplätzen, Immundeterminanten pro Molekül; (5)

m = 10^6: Erythrocyt mit 10^6 identischen Bindungsplätzen, Immundeterminanten, vom (Blutgruppen-) Typ A pro 150 μm^2.

n = 2: Immunglobulin G (M_r = 150000) mit 2 identischen Bindungsplätzen pro Molekül; Abstand etwa 10 nm

n = 10: Immunglobulin M (M_r = 900000) mit 10 identischen Bindungsplätzen pro Molekül; Abstand bis etwa 30 nm.

Die Besetzung eines der Bindungsplätze der Art ^nQ kann die n − 1 restlichen Bindungsstellen des Aggregates für den gleichen Reaktionspartner unbeeinflußt lassen (Unabhängigkeit der Bindungsstellen) oder die restlichen Bindungsstellen so beeinflussen (homotrope Kooperativität), daß entweder deren Affinität größer wird (positive homotrope Kooperativität) oder kleiner wird (negative homotrope Kooperativität). Ordnungen von P_i oder Q_j, die meßbare Signale geben (Indikatoren) werden mit * gekennzeichnet. Besonders für diese gelten Definitionsgleichungen und Ableitungen folgender Art (Beispiel P*, markiertes Antigen; für Q*, markierte Antikörper, kann Analoges definiert werden):

$$[P^*] \equiv F;\ [P^*Q] \equiv B;\quad p^* \equiv [P^*] + [P^*Q] \equiv T;$$

$$\frac{B}{F} \equiv R;\quad \frac{B}{T} = \frac{R}{1+R};\ r \equiv \frac{B}{q}$$

F freie Bindungsplätze;
B besetzte Bindungsplätze;
T Gesamtzahl der eingesetzten Bindungsplätze
R Response, Wirkung einer bestimmten Dosis p oder q auf die Verteilung des „Indikators"
r Anteil der besetzten Bindungsplätze an der Gesamtzahl der Bindungsplätze ($r' \equiv B/p$).

2. Reaktions-Energien

In einem System kann eine Assoziation von P_i und Q_j prinzipiell dann stattfinden, wenn

1. einzelne Elemente (z.B. Moleküle) des Systems hinreichend große Energien besitzen, deren Summe in einer Größe $E \geqslant E_0$ (Schwellenenergie) zusammengefaßt wird, und wenn diese Moleküle hinreichend häufig richtige Orientierungen zueinander eingehen, deren Wahrscheinlichkeit in einer Größe A enthalten ist. E und A können mit Hilfe der experimentell zugänglichen Reaktions-Geschwindigkeitskonstante k charakterisiert werden (11).

2. die mit der Reaktion verbundene Änderung der *Gibbs*schen Energie ΔG, der Differenz aus Reaktions-Enthalpie-Glied ΔH und Reaktions-Entropie-Glied $T\Delta S$, des gesamten Systems einen negativen Wert erhält ($\Delta G < 0$). ΔG, ΔH und ΔS können mit Hilfe der experimentell zugänglichen Konzentrationen der Reaktionspartner und deren Quotient K charakterisiert werden. Ein Reaktionsgleichgewicht ist dann erreicht, wenn die *Gibbs*sche Funktion ein Minimum erreicht hat (12).

Die Aussagen 1) und 2) sind in Gleichung 1 enthalten.

Zu 1): Die Theorie der molekularen Übergangszustände (z.B. $P_iQ_j^*$ in $P_i + Q_j \rightleftarrows P_iQ_j^* \rightleftarrows P_iQ_j$) führte zu Gl. 3; die Theorie der Molekül-Kollisionen ergab ähnliche Ausdrücke (siehe z.B. *J. H. Knox*, Molecular Thermodynamics, Chichester 1978).

Die strukturelle Analogie von Gleichung 3 zur empirischen mit üblichen Labortechniken experimentell nachprüfbaren Gleichung 4 ist offensichtlich. Des-

wegen kann Gleichung 3 zur Deutung, Gleichung 4 zur Bestimmung der unbekannten Parameter herangezogen werden (11, 13, 14).

$$\text{(Gl. 3)} \quad k = \frac{\bar{k}T}{h} e^{\frac{\Delta S_{Akt}}{R}} e^{-\frac{\Delta H_{Akt}}{RT}}$$

$$\text{(Gl. 4)} \quad k = A_{exp}\, e^{-\frac{E_{exp}}{RT}}$$

$$\ln k = \ln A_{exp} - \frac{E_{exp}}{RT} \qquad \textit{Arrhenius}\text{-Gleichung}$$

k: k_{+1ij} oder k_{-1ij} von Gleichung 1
h: *Planck*-Wirkungsquantum $6{,}6 \times 10^{-34}$ Js
ΔS_{Akt}: Entropie der Aktivierung
ΔH_{Akt}: Enthalpie der Aktivierung
A_{exp}: Präexponentialfaktor
E_{exp}: experimentelle Aktivierungsenergie
$\bar{k}$, T, e, R: s. Gl. 1, Legende

Zur Ermittlung von A_{exp} und E_{exp} werden im allgemeinen die Werte der Reaktions-Geschwindigkeitskonstanten k bei verschiedenen Temperaturen bestimmt. Diese liegen im *Arrhenius*-Diagramm (ln k vs. 1/T) nach Gleichung 4 auf einer Geraden. In der Praxis gilt der Anstieg der Geraden als Quotient E_{exp}/R und der Ordinatenabschnitt bei $1/T \rightarrow 0$ als $\ln A_{exp}$. Da zumeist von Reaktionen mit Proteinen nur die k-Werte eines engen Temperaturbereiches eingesetzt werden können, sind insbesondere die durch Extrapolation gewonnenen Werte von A_{exp} mit großen Fehlern behaftet. Die ermittelten Werte sind innerhalb des Meßbereiches und innerhalb der Meßgenauigkeit konstant, d.h. anscheinend unabhängig von der Temperatur, wogegen die Theorie eine Temperaturabhängigkeit der A_{exp} und E_{exp} zugrundeliegenden molekularen Werte einschließt.

Zur Deutung von E_{exp} und A_{exp} bzw. zur Bestimmung von ΔH_{Akt} und ΔS_{Akt} werden die Gleichungen 3 und 4 verglichen; offensichtlich entspricht die Aktivierungs-Enthalpie ΔH_{Akt} dem Wert $E_{exp} - RT$ und die Aktivierungs-Entropie ΔS_{Akt} dem Wert $R \ln (A_{exp} N h/RT) - R$. Aus der Gleichung folgt fernerhin, daß die Geschwindigkeit einer Reaktion um so größer ist, je kleiner die Enthalpiezunahme und je größer die Entropiezunahme beim Übergang vom Grundzustand $P_i + Q_j$ oder P_iQ_j zum Übergangszustand $P_iQ_j^*$ der Reaktion ist.

Ein großes positives Enthalpieglied wird als Dehnung oder Stauchung chemischer Bindungen bei Ausbildung des Übergangszustandes gedeutet. Ein großes negatives Entropieglied gilt als Ausdruck der geringen Wahrscheinlichkeit der Ausbildung präziser komplementärer Konformationen und Berührungswinkel der Reaktanden P_i und Q_j. Von Protein-Bindungsreaktionen wird angenommen, daß sich die Aktivierungsentropie ΔS_{Akt} nicht wesentlich von der Reaktionsentropie ΔS (siehe Gleichung 5) unterscheidet, d.h., daß die Entropie von $P_iQ_j^*$ der Entropie von P_iQ_j etwa gleich ist. Die wenigen bisher vorliegenden experimentellen Befunde sind nicht ganz einheitlich. Aus der Tatsache, daß k_{+1} kaum temperaturabhängig ist und zumeist nicht wesentlich unter den für diffusionskontrollierte Reaktionen zu erwartenden Werten liegt (10^9 l $mol^{-1}s^{-1}$), wurde geschlossen, daß zur Assoziation von Liganden an Proteine keine großen Aktivierungsenergien benötigt werden (13, 14); Beispiele für offenbar nicht-diffusionskontrollierte Assoziationsreaktion siehe z.B. l.c. (6), (15). Die Aktivierungsenergie zur Dissoziation steroidaler Haptene von Antikörpern lag mit 42—63 kJ/mol (10—15 kcal/mol) deutlich unter dem für die Dissoziation von Cortisol-Transcortin gemessenen Wert von 151 kJ/mol (36 kcal/mol). Für diesen Komplex ist auch eine relativ hohe Aktivierungsenergie der Assoziation gemessen worden (109 kJ/mol = 26 kcal/mol). Dagegen wird die Konformation eines Immunglobulins durch die Assoziation eines steroidalen Haptens offenbar nur geringfügig verändert (6, 16, 17).

Zu 2): Die mit Gleichung 1 definierte Änderung der *Gibbs*schen Standardenergie ΔG° gilt für Standardreaktionen. Die allgemein mit Fortschreiten einer Reaktion verbundene Änderung der *Gibbs*schen Energie ΔG wird durch die Gleichungen 5 und 6 festgelegt.

Nach Gleichung 5 wird die Negativität von ΔG umso größer und somit auch die Triebkraft einer Reaktion um so größer, je kleiner die Enthalpiezunahme ist (durch größeres negatives ΔH) und je größer die Entropiezunahme ist (durch größeres positives $T\Delta S$); sie ist abhängig von den Ausgangs-Konzentrationen der Reaktionspartner (zum chemischen Potential des gebundenen Liganden, dG/dn, siehe l.c. (18)).

Zur Normierung der in Gleichung 5 und Gleichung 6 enthaltenen Größen ΔG, ΔH und ΔS wurden die Standardreaktionen definiert, an deren Beginn alle Reaktionspartner in der Konzentration 1 (z.B. 1 mol/l) und an deren Ende alle Reaktionspartner in den jeweiligen Gleichgewichtskonzentrationen vorliegen. Durch diese Normierung wird $\ln K_{ini} = \ln 1 = 0$ und $\ln K_{fin} = \ln K$ (K = Gleichgewichtskonstante) und somit Gleichung 6 zu Gleichung 7 und Gleichung 8, den logarithmierten Formen der Gleichung 1. Die zugehörigen Potentialdifferenzen werden als Standardpotentiale ΔG°, ΔH° und ΔS° bezeichnet. Durch Differenzierung wird Gleichung 9a erhalten. Gleichung 9a kann integriert werden, wenn im inter-

essierenden Bereich ΔH° von der Temperatur unabhängig ist. Als Folge davon erhält man die Gleichung 9b, ein Analogon der *Arrhenius*-Gleichung (Gl. 4).

(Gl. 5) $\Delta G = \Delta H - T\Delta S$ *Gibbs-Helmholtz*-Gleichung

(Gl. 6) $\Delta G = RT \ln \frac{K_{ini}}{K_{fin}}$

(Gl. 7) $\Delta G^\circ = - RT \ln K = - RT \ln \frac{k_{+1}}{k_{-1}}$

(Gl. 8) $\ln K = - \frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}$

(Gl. 9a) $\frac{d \ln K}{dT} = -\frac{\Delta H^\circ}{RT^2}$ *van't Hoff*-Gleichung

(Gl. 9b) $\ln K = - \Delta H^\circ \frac{1}{RT} + C$

(Gl. 10) $\frac{d\Delta H^\circ}{dT} = \Delta Cp^\circ$ *Kirchoff*-Gleichung

ΔG: Änderung der *Gibbs*-Energie
ΔH: Änderung der Enthalpie
ΔS: Änderung der Entropie
ΔCp: Änderung der Wärmekapazität
K_{ini}: Quotient der initialen Konzentrationen der Reaktionspartner $[\ ]_{ini}$
K_{fin}: Quotient der finalen Konzentrationen der Reaktionspartner $[\ ]_{fin}$
Zähler der Quotienten: kontinuierliche Multiplikation der Konzentrationen der Reaktionsprodukte; stöchiometrische Koeffizienten als Exponenten.
Nenner der Quotienten: kontinuierliche Multiplikation der Konzentrationen der Reaktanden; stöchiometrische Koeffizienten als Exponenten.
z.B. für: $1P + 1Q \rightleftarrows 1PQ$

$$K_{ini} = \frac{[PQ]^1_{ini}}{[P]^1_{ini}\,[Q]^1_{ini}}$$

$$K_{fin} = \frac{[PQ]^1_{fin}}{[P]^1_{fin}\,[Q]^1_{fin}}$$

Die Zahlenwerte für ΔG° (bzw. ΔG) lassen sich unmittelbar aus den Werten von K (bzw. von K_{ini} und K_{fin}) errechnen (Gl. 6 bzw. Gl. 7). Theoretische Schätzung über *Van der Waals*-Interaktion/*Hamaker*-Konstanten der Assoziation von P und Q in der Gasphase mit nachfolgender Hydratation führt als kleine Differenz großer Zahlen erwartungsgemäß nur ungefähr in den Bereich der experimentell ermittelten ΔG° Werte (8).

Die Zahlenwerte von ΔH° lassen sich nach Gleichung 9b aus der Temperaturabhängigkeit der K-Werte ermitteln. Hiernach liegen die K-Werte im *van't Hoff*-Diagramm (ln K vs. 1/T) auf einer Geraden. In der üblichen Laborpraxis gilt der Anstieg der Geraden als Quotient $\Delta H^\circ/R$. Da aber im strengen Sinne sowohl ΔH° wie auch ΔS° von der Temperatur abhängen, sind die Voraussetzungen für den Übergang von Gleichung 9a zu Gleichung 9b nicht gegeben und im *van't Hoff*-Diagramm ist ein nicht-linearer Verlauf der K-Werte zu erwarten. In der temperaturabhängigen Änderung von ΔH° drückt sich ΔCp aus, der Unterschied der Summen der spezifischen Wärmen von Reaktanden und Produkten (*Kirchoff*-Satz). Die Zahlenwerte von ΔH (= Wärmetönung der Reaktion) lassen sich auch, und bei Temperaturabhängigkeit von ΔH richtiger, direkt im Kalorimeter bestimmen. Läßt man die Reaktion im Kalorimeter bei verschiedenen Temperaturen ablaufen, kann man aus der Temperaturabhängigkeit der Wärmetönung den Wert von ΔC_p° berechnen, bei linearem Verlauf als $\Delta H^\circ/\Delta T$, sonst über quadratische Regression (z.B. $\Delta H^\circ = A + BT + CT^2$; $C_p^\circ = B + 2CT$).

Die Zahlenwerte von ΔS° werden nach Gleichung 5 durch Differenzenbildung aus den Werten von ΔG° und ΔH° berechnet oder aus dem Achsenabschnitt, in Analogie zur Aktivierungsentropie (Gl. 4).

Für konkrete Reaktionen sind die numerischen Werte der Potentiale zumindest näherungsweise experimentell bestimmbar. Wegen der unvermeidlichen Abweichung der experimentellen Bedingungen von „Standardbedingungen" (z.B. $\ln [H^+] = 0$!) können nur „apparente" Werte ermittelt werden, was durch z.B. $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$, und $\Delta S^{\circ\prime}$ gekennzeichnet werden sollte; die experimentellen Bedingungen sind anzugeben (z.B. 25 °C (298,15 K); pH 7,6 Ionenstärke 0,1 mol/l) (19).

Die *Gibbs*schen Potentiale der Immunreaktionen liegen bei −21 bis −63 kJ/mol (−5 bis −15 kcal/mol). Immunreaktionen erwiesen sich zum Teil als überwiegend Enthalpie-getrieben, zum Teil als überwiegend Entropie-getrieben; es wurden sowohl positive wie auch negative Werte für ΔC_p° publiziert (4, 6, 13, 20). Auch die Frage, ob die Dispersion der ΔG°-Werte einer „natürlichen" Antikörperpopulation auf die Streuung des Enthalpie- oder des Entropie-Gliedes zurückzuführen ist, bleibt offen, auch wenn zuletzt das Enthalpie-Glied als Ursache der Streuung von ΔG° favorisiert wurde (21). Zumindest an einigen Hapten-Antikörper-Paaren wurde eine Enthalpie-Entropie-Kompensation nachgewiesen. Mit steigender Temperatur ging der Einfluß des Enthalpie-Anteils zugunsten des Entropie-Anteils zurück (22).

Die Deutung von $\Delta H°$, $\Delta S°$ und $\Delta C_p°$ als Ergebnis molekularer Vorgänge muß Änderungen der Reaktanden und des Lösungsmittels berücksichtigen, da sich mit der Reaktion auch die Solvatationszustände ändern (statt Formel 1 besser:)

$$P_{i(x + m\ H_2O)} + Q_{j(y + n\ H_2O)} \rightleftarrows P_iQ_{j(m + n\ H_2O)} + (x + y)\ H_2O$$

Die vorliegenden Deutungen sind unvollständig, da die thermodynamischen Potentiale die Summe aller molekularen Prozesse repräsentieren und da der Nachweis von Änderungen einzelner Molekülspezies mit Hilfe von z.B. Röntgen- oder NMR-Spektroskopie noch nicht abgeschlossen ist. Lediglich eine struktur-unabhängige und somit unspezifische Komponente des Entropie-Terms, eine Mischungsentropie, kann aus der Standard-Entropie-Änderung $\Delta S°$ einfach eliminiert werden. Hierdurch werden Werte für die „unitarische" Entropie-Änderung ΔS_u und für die entsprechend abgeleitete „unitarische" Änderung des *Gibbs*schen Potentials ΔG_u erhalten, mit denen die Stabilität des Komplexes besser charakterisiert werden kann.

$$\Delta S_u = \Delta S° + R \ln 1/[H_2O]$$

$$\Delta G_u = \Delta G° - R \ln 1/[H_2O]T$$

$(R \ln 1/[H_2O] = 33{,}5\ J\ mol^{-1}\ K^{-1} = 7{,}98\ cal\ mol^{-1}\ K^{-1}$
(3, 7, 8, 12, 14, 22, 23).
$[H_2O] = 55{,}6\ mol/l$

Der Energiegewinn, der mit der Assoziationsreaktion verbunden ist, d.h. die Negativität von $\Delta G°$, wird auf die Summation zahlreicher, im einzelnen schwacher und z.T. gegensinnig wirkender Kräfte zurückgeführt, die aus den Wechselwirkungen verschiedener Molekülgruppen resultieren, überwiegend sind dies die *van der Waals*-Kräfte, die Wasserstoffbrücken-Bindungen und die hydrophoben Wechselwirkungen, d.h. die Minimierung der Exposition unpolarer Gruppen und die Maximierung der Exposition polarer und ionischer Gruppen.

Beim Lösen unpolarer Gase in Wasser (25 °C) wird wegen der Neuorientierung der Wassermoleküle an den Grenzflächen (Ausbildung „eisartiger" Strukturen) die negative Entropie-Änderung das *Gibbs*sche Potential bestimmen und wegen der Ausbildung von Wasserstoff-Brücken wird eine positive Änderung der Wärmekapazität erwartet („Schmelzen" der eisartigen Strukturen). Beim Lösen von Ionen in Wasser wird wegen der Ion-Dipol-Wechselwirkung die positive Enthalpie-Änderung das *Gibbs*sche Potential bestimmen und wegen der Verminderung von Wasserstoff-Brücken wird eine negative Änderung der Wärmekapazität erwartet. Die Umkehr derartiger Effekte bei der Assoziation von P und Q im Bereich der Kontaktregionen von P und Q, die zusätzlichen intermolekularen Wechselwirkungen zwischen P und Q und die Energiebeiträge durch intramolekulare Konformationsänderungen von P und Q ergeben als Summe die meßbaren Änderungen $\Delta G°$, $\Delta H°$, $\Delta S°$ und $\Delta C_p°$. Die oben genannte Enthalpie-Entropie-Kompensation könnte wie folgt verlaufen. Bei niedrigen Temperaturen wird die negative Gesamt-Enthalpie- und -Entropie-Änderung überwiegend durch die „intrinsische" Enthalpie- und Entropie-Änderung der Komplexbildung bestimmt. Die mit steigender Temperatur zunehmende Verminderung der Negativität mit Übergang zu positiven Werten der Gesamtpotentiale wäre eine Folge der Enthalpie- und Entropie-Änderung durch Vorgänge im Lösungsmittel. Aus dem gegensinnigen Einfluß der Enthalpie- und Entropie-Änderungen resultiert eine im Meßbereich ungefähr konstante Änderung des *Gibbs*schen Potentials.

3. Reaktions-Geschwindigkeitskonstanten und Reaktions-Gleichgewichtskonstanten

Die Berechnung von A_{exp}, E_{exp}, $\Delta G°$, $\Delta H°$ und $\Delta S°$ setzt die Kenntnis der Geschwindigkeitskonstanten k_{+1}, k_{-1} und/oder die Kenntnis der Gleichgewichtskonstanten $K = k_{+1}/k_{-1}$ bei verschiedenen Temperaturen voraus. Die Größen k und K können unabhängig voneinander experimentell bestimmt werden, k über die Reaktionsgeschwindigkeiten, K über die Gleichgewichts-Konzentrationen. Als Quotient von k_{+1} und k_{-1} kann K auch über die Reaktionsgeschwindigkeiten ermittelt werden. Da beide Verfahren mit systematischen experimentellen Fehlern behaftet sein können, empfiehlt sich der Vergleich der nach beiden Verfahren gewonnenen Ergebnisse. Für eine subtilere Behandlung der Reaktionskinetik auch mehrwertiger und trägerfixierter Reaktionspartner mit Unterscheidung der diffusiven und reaktiven Anteile von k und K siehe z.B. l.c. (24).

Reaktions-Geschwindigkeitskonstanten

Die durch Formel 1 definierte reversible Reaktion setzt sich aus den Partialreaktionen nach Formel 1′ und 1″ zusammen.

(Formel 1′) $P_i + Q_j \xrightarrow{k_{+1ij}} P_iQ_j$

(Formel 1″) $P_iQ_j \xrightarrow{k_{-1ij}} P_i + Q_j$

[ ] bedeutet im folgenden „Konzentration zur Zeit t", also nicht mehr „Gleichgewichtskonzentration" $[\ ]_{äq}$.

Wird die Reaktion nach Formel 1′ gestartet, findet zur Zeit t_o ausschließlich die Assoziationsreaktion statt ($v_{-1} = 0$), deren Geschwindigkeit v_{+1} durch Gleichung 11 definiert, zur Zeit t_o maximal ist.

$$\text{(Gl. 11)} \quad v_{+1} = -\frac{d[P]}{dt} = -\frac{d[Q]}{dt} = \frac{d[PQ]}{dt} = k_{+1}[P]\,[Q]$$

Wird die Reaktion nach Formel 1″ gestartet, findet zur Zeit t_o ausschließlich die Dissoziationsreaktion statt ($v_{+1} = 0$), deren Geschwindigkeit v_{-1}, durch Gleichung 12 definiert, zur Zeit t_o maximal ist.

$$\text{(Gl. 12)} \quad v_{-1} = -\frac{d[PQ]}{dt} = \frac{d[P]}{dt} = \frac{d[Q]}{dt} = k_{-1}[PQ]$$

Zu irgendeinem Zeitpunkt zwischen t_o und $t_{äq}$ sind beide Geschwindigkeiten, v_{+1} und v_{-1} von Null verschieden und es gilt Gleichung 13 (Analytische Lösung: l.c. (26).

$$\text{(Gl. 13)} \quad \frac{d[PQ]}{dt} = -\frac{d[P]}{dt} = -\frac{d[Q]}{dt} = k_{+1}[P]\,[Q] - k_{-1}[PQ] \neq 0$$

Zur Zeit $t_{äq}$ hat sich das kinetische Gleichgewicht der Reaktion eingestellt. Von diesem Zeitpunkt an gilt Gleichung 14 a–c.

$$\text{(Gl. 14a)} \quad \frac{d[PQ]}{dt} = -\frac{d[P]}{dt} = -\frac{d[Q]}{dt} = k_{+1}[P]\,[Q] - k_{-1}[PQ] = 0$$

$$\text{(Gl. 14b)} \quad k_{+1}[P]\,[Q] = k_{-1}[PQ]$$

$$\text{(Gl. 14c)} \quad \frac{[PQ]}{[P]\,[Q]} = \frac{k_{+1}}{k_{-1}} = K$$

Zur experimentellen Bestimmung einer der beiden Geschwindigkeitskonstanten der Gleichung 13 kann durch geeignete Versuchsbedingung die jeweils gegenläufige Reaktion unterdrückt werden, so daß nach Gleichung 11 oder Gleichung 12 gerechnet werden kann (6, 25, 26, siehe aber auch l.c. 24).

Bestimmung von k_{+1}

Unter der Voraussetzung $v_{+1} \gg v_{-1}$ ist k_{+1} schwieriger zu bestimmen als k_{-1}. Als maximale Assoziationsgeschwindigkeit gilt die diffusionskontrollierte Reaktion mit $k = 10^9$ l mol^{-1}s^{-1} ($t_{1/2} = \frac{1}{k[P]_o} = 10^{-9}$ s bei Konzentrationen von 1 mol/l). Im allgemeinen liegen Bestimmungen dieser Art außerhalb der Möglichkeiten der üblichen Labortechnik; es werden z.B. Strömungs- und Relaxationsmethoden notwendig (16, 27). Bei niederen Konzentrationen und hohen Affinitäten der Reaktanden ist es jedoch möglich, mit den üblichen Labormethoden auch für die Geschwindigkeitskonstanten der Assoziationsreaktionen Näherungswerte zu erhalten, selbst wenn kein „inneres Signal" der Moleküle eine Unterscheidung von P* und P*Q ermöglicht, sondern zu ihrer Bestimmung eine Trennung dieser Molekülspezies erforderlich ist (6, 25).

Wenn $v_{+1} \gg v_{-1}$, dann kann, zumindest in der Initialphase der Reaktion, statt Gleichung 13 die Gleichung 11 verwendet werden. Sie wird zur Gleichung 15 umgewandelt.

$$\text{(Gl. 15)} \quad \frac{d[PQ]}{dt} = k_{+1}(p - [PQ])\,(q - [PQ])$$

$$k_{+1}\,dt = \frac{d[PQ]}{(p - [PQ])\,(q - [PQ])}$$

Durch Integration erhält man die Gl. 16 und 17 (25).

$$\text{(Gl. 16)} \quad k_{+1}\,t = \frac{1}{q - p} \ln \frac{p(q - [PQ])}{q(p - [PQ])}$$

$$\text{(Gl. 17)} \quad k_{+1}\,t(q - p) = \ln \frac{p}{q} + \ln \frac{q - [PQ]}{p - [PQ]}$$

Gleichung 17 zeigt, daß die Steigung der beim Auftragen von

$$\ln \frac{q - [PQ]}{p - [PQ]}$$

gegen die Zeit t erhaltenen Geraden $k_{+1}(q - p)$ beträgt. Es ist also prinzipiell möglich, k_{+1} zu bestimmen, wenn die Konstanten p und q bekannt sind und die Veränderung von PQ zuverlässig bestimmt werden kann.

Wählt man im Experiment ein Verhältnis $p/q \approx \geqslant 10$ oder $\approx \leqslant 1/10$, dann wird von Gleichung 15 der Ausdruck $p - PQ \approx p$ oder es wird der Ausdruck $q - PQ \approx q$: die Meßdaten können dann als Daten einer pseudo-monomolekularen Reaktion behandelt werden (siehe Gl. 18).

Weichen die transformierten Meßdaten von der Linearität ab, kann die Richtigkeit des gewählten Modells überprüft werden; in Anbetracht der Schnellig-

keit der Reaktion dürften Fehler in der Versuchsanordnung oder Messung als Ursache für die Abweichung von der Linearität wahrscheinlicher sein. Es kann auch auf eine apparente Geschwindigkeitskonstante k_{app} übergegangen werden:

$$k_{app}\ t = \frac{B_t}{B_{äq}\ (B_{äq} - B_t)} \qquad (28).$$

Bestimung von k_{-1}

Da nach Voraussetzung $v_{-1} \ll v_{+1}$, muß die Rück-Assoziation P + Q → PQ unterbunden werden. Wenn dies möglich ist (kompetitive Hemmung der Bindung von P, Adsorption von P), kann von Formel 1'' und somit auch von Gleichung 12 ausgegangen werden; Gleichung 12 wird umgeformt zu Gleichung 18 (siehe z.B. l.c. (6), (25), (26)).

$$\text{(Gl. 18)} \quad k_{-1}\ dt = -\frac{d[PQ]}{[PQ]};$$

$$k_{-1}\ t = -\ln [PQ]_t - \ln [PQ]_o = -\ln \frac{[PQ]_t}{[PQ]_o}.$$

Die Gleichung 18 zeigt, daß beim Auftragen des logarithmierten Quotienten (PQ-Konzentrationen zu verschiedenen Reaktionszeiten t dividiert durch die PQ-Konzentration zur Zeit t = 0) gegen die Reaktionszeit t eine Gerade mit der Steigung k_{-1} erhalten wird.

Weichen die transformierten Meßdaten von der Linearität ab und können Fehler in der Versuchsanordnung und Messung ausgeschlossen werden, so ist zu prüfen, ob nicht statt $Q_j = Q$, z.B. das Modell Q_j (j = 1, 2, . . . n) den Daten besser anzupassen ist (26, 28). Über scheinbare negative Kooperativität siehe l.c. (24). Wenn es die Dissoziations-Geschwindigkeitskonstante ist, die den Wert der Gleichgewichts-Konstante im wesentlichen bestimmt, dann liegen die Werte für k_{-1} von Immunkomplexen zwischen 10^{-5} s^{-1} (eine Dissoziation pro Monat) und 10^{6} s^{-1} (eine Dissoziation pro µs) (14).

Reaktions-Gleichgewichtskonstanten

Zur Berechnung der Gleichgewichtskonstanten aus bekannten Größen (z.B. p) und den gemessenen Konzentrationen (z.B. B, F) sind Vorkenntnisse über Eigenschaften der Reaktionspartner erforderlich. Aus diesen Vorkenntnissen werden Modellvorstellungen entwickelt und es wird geprüft, wie weit das hypothetische Modell mit den Meßwerten in Einklang zu bringen ist. Als diagnostisches Hilfsmittel empfiehlt sich die Transformation der Gleichung 1, mit i = 1, j = 1,

a) nach *Langmuir-Michaelis-Menten*

$$B = \frac{q\ K\ F}{1 + K\ F}$$

b) nach *Scatchard*

$$r/F = -\ Kr + K$$

c) nach *Sips-Hill-Nernst*

$$\ln(B/(q - B)) = \alpha \ln K + \alpha \ln F$$

Aus der Lage der Meßpunkte in den Diagrammen B vs. F, r/F vs. r, ln(B/(q − B)) vs. F, wird auf Abweichungen vom einfachsten Modell, i = 1, j = 1, K_{ij} = K, geschlossen; je nach Plausibilität wird eines der folgenden Modelle den realen Versuchsbedingungen unterlegt (26, 29, 30).

1. Modell: m Ordnungen von unabhängigen Antigen-Bindungsplätzen
n Ordnungen von unabhängigen Antikörper-Bindungsplätzen
(Modell konkurrierender Reaktionen)

Die in Gleichung 1 enthaltenen n × m Gleichungen und die in Gleichung 2 enthaltenen n + m Gleichungen lassen sich durch Substitution von $[P_i\ Q_j]$ in Gleichung 2 komprimieren zu den n-Gleichungen der Gleichung 2a oder zu den m-Gleichungen der Gleichung 2b, mit denen die n × m Gleichgewichte der Matrix (Abb. 1) determiniert sind.

Zur weiteren Rechnung wird die Gleichung bevorzugt, die die wenigsten Indices beinhaltet. Wenn also m < n, wird Gleichung 2a bevorzugt und zur Gleichung 19 umgewandelt (31).
Da

$$R = \frac{B}{F} = \frac{T - F}{F} = 1 + \frac{T}{F},$$

gilt die *Scatchard* Transformation (Gl. 19).

$$\text{(Gl. 19)} \quad R_i = \frac{p_i}{[P_i]} - 1 = \sum_{j=1}^{n} \frac{K_{ij}\ q_j}{1 + \sum_{a=1}^{m} K_{aj}\ [P_a]}$$

Die Parameter p_i, q_j und K_{ij} können mit Hilfe elektronischer Datenverarbeitung an die Ergebnisse experimenteller Versuchsreihen angepaßt werden, die im allgemeinen aus den Gleichgewichts-Konzentra-

tionen eines markierten Antigens bestehen, dessen Dosis p_i variiert wird. Gleichung 19 kann nicht analytisch gelöst werden, sondern nur numerisch z.B. mit Hilfe von Iterationsverfahren. Da die Anzahl berechenbarer Parameter durch die Anzahl informativer Meßpunkte begrenzt ist, können selten für mehr als 5 Parameter Zahlenwerte ermittelt werden, für die bereits über 25 Meßpunkte gefordert werden (26, 29).

2. Modell: m = 1 Ordnung von Antigen-Bindungsplätzen
n Ordnungen von Antikörper-Bindungsplätzen
(Modell zur Charakterisierung einer Antikörper- oder, allgemeiner, einer „Rezeptor"Population)

Wenn P_i zu P oder P* wird, vereinfacht sich Gleichung 19 zu Gleichung 19b.

$$\text{(Gl. 19b)} \quad R = \sum_{j=1}^{j=n} \frac{K_j\, q_j}{1 + K_j[F]} = \frac{K_1\, q_1}{1 + K_1[F]} + \frac{K_2\, q_2}{1 + K_2[F]} + \ldots + \frac{K_n\, q_n}{1 + K_n[F]}$$

a) Wenn das System neben den Antikörper-Bindungsplätzen („spezifische Binder") einen „Binder" Q_m mit niedriger Affinität und hoher Kapazität (z.B. „unspezifische Adsorption") enthält, dann wird

$K_n[F] \rightarrow 0$ und somit $K_n q_n/(1 + K_n[F])$ zur Konstanten $K_n q_n = N$

b) Wenn für das System eine Ordnung von Antikörper-Bindungsplätzen mit einer mittleren Bindungsenergie

$$\Delta\bar{G}^\circ = \bar{R}T \ln \bar{K}$$

$$\bar{K} = \sum_{j=1}^{n} \frac{q_j\, K_j}{\sum_{j=1}^{n} q_j}$$

definiert werden kann, dann verteilen sich alle ΔG_j° symmetrisch um $\Delta\bar{G}^\circ$. Das Maß für die Breite der Verteilung, d.h. für die Heterogenität der Antikörper, ist

σ, wenn eine *Gauss*-Verteilung angenommen wird
α, wenn eine *Sips*-Verteilung angenommen wird.

Die Annahme einer *Gauss*-Verteilung führt zu Gleichung 20, die Annahme einer *Sips*-Verteilung zu Gleichung 21 (32).

$$\text{(Gl. 20)} \quad r = 1 - \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\beta^2}}{1 + \bar{K}\, Fe^{\beta\sigma}}\, d\beta; \quad \beta = \frac{\ln \frac{K}{\bar{K}}}{\sigma}$$

$$\text{(Gl. 21)} \quad r = \frac{\bar{K}F^\alpha}{1 + \bar{K}F^\alpha}; \quad \ln \frac{r}{1 - r} = \alpha\, K\, F; \quad (\alpha = 0 \leqslant \alpha \leqslant 1)$$

Sips-Hill Transformation

Zusammenhang zwischen σ und α:

$$\sigma = 2\sqrt{\pi} \cot\left(\frac{\pi\alpha}{2}\right)$$

Konkrete Bedeutung von σ und α:

α	σ	Bereich mit 75% der Bindungsstellen
0,5	4,3	0,0025 bis 40 $\bar{K}$
0,7	2,4	0,16 6
0,8	1,8	0,27 3,7
1,0	0,0	alle Bindungsstellen 1 × $\bar{K}$ d.h. $K_j = \bar{K}$

Zur Berechnung des Heterogenitäts-Index α wird nach Gleichung 21 $\ln(r/(1 - r))$ gegen F aufgetragen (*Sips-Hill*-Diagramm); hierzu muß q bereits bekannt sein, ggf. aus dem *Scatchard*-Diagramm ermittelt sein. Der Anstieg der Geraden ergibt α; zur Bedeutung der Asymptoten bei nicht-linearem *Sips-Hill*-Graph, siehe z.B. l.c. (30); neuere Behandlung der Verteilungsfunktion siehe z.B. l.c. (9).

Für jedes α und σ gilt, daß die mittlere intrinsische Assoziationskonstante $\bar{K}$ den reziproken Wert von [P] bei Halbsättigung von q (bzw. n × q) entspricht; dieser Wert ist im *Scatchard*-Diagramm unmittelbar zugänglich.

c) Wenn die Bindungsplätze P_i und/oder Q_j des Antigens und/oder des Antikörpers zu Aggregaten zusammengefaßt sind, z.B. mehrwertiges Antigen und/oder mehrwertiger Antikörper, deren Bindungsplätze sequentiell besetzt werden, dann können deren intrinsische Affinitäten und damit deren intrinsische Gleichgewichtskonstanten $\overset{j}{K}$ identisch oder nicht-identisch sein. Bei ungleichen intrinsischen Affinitäten kann die Verschiedenheit von vornherein vorgegeben sein oder durch die vorangegangene Bindung induziert worden sein.

Im folgenden wird $Q(^nQ)$ als mehrwertig, n wertig, behandelt; die Indices (j = 1, 2 . . . n) von P bedeutend abweichend vom bisherigen Gebrauch die stöchiometrischen Faktoren und nq die Gesamtzahl der Bindungsplätze.

(Gl. 22)

$$P + {}^{n}Q = P^{n}Q; \quad K_1 = n \overset{1}{K};$$

$$P + P^{n}Q = P_2{}^{n}Q; \quad K_2 = \frac{n-1}{2} \overset{2}{K};$$

. . . . = . . .; . = ...;

$$P + P_{j-1}{}^{n}Q = P_j{}^{n}Q; \quad K_j = \frac{n-j+1}{j} \overset{j}{K};$$

. . . . = . . .; . = ...;

$$P + P_{n-1}{}^{n}Q = P_n{}^{n}Q; \quad K_n = \frac{n-n+1}{n} \overset{n}{K};$$

Bei n wertigen Einheiten wird das Verhältnis der „experimentellen" Gleichgewichtskonstanten K_j zu den „intrinsischen" Gleichgewichtskonstanten $\overset{j}{K}$ durch den „statistischen" Faktor ausgedrückt (Gl. 23).

(Gl. 23) $$\frac{n-j+1}{j} = \frac{K_j}{\overset{j}{K}}$$

Statistischer Faktor: Die Wahrscheinlichkeit, daß P mit einer n-wertigen Einheit, von deren n Bindungsstellen mit $\overset{j}{K} = K$ noch keine besetzt ist, an einer der n Stellen assoziiert, ist n-mal wahrscheinlicher, als daß nach Besetzung von n − 1 Stellen die letzte, also die n^{te} Stelle besetzt wird. Deswegen wird K_1 zu nK.

Die Wahrscheinlichkeit, daß P von einer n-wertigen Einheit, von deren n Bindungsstellen mit $\overset{j}{K} = K$ alle mit P besetzt sind, $P_n{}^{n}Q$, von einer der n Stellen abdissoziiert, ist n-fach wahrscheinlicher, als daß P von einer ${}^{n}Q$ Einheit abdissoziert, von der erst eine der n Stellen besetzt ist. Deswegen wird K_n zu 1/n K.

Die Gruppe Gleichung 22 kann unter Verwendung von Gleichung 23 zur *Adair*-Funktion (Gl. 24) transformiert werden (14, 30).

(Gl. 24) $$r = \frac{\sum_{j=1}^{n} j\,\psi_j\,F^j}{1 + \sum_{j=1}^{n} \psi_j\,F^j}$$ *Adair*-Gleichung

ψ: *Adair*-Konstanten, deren molekular-physikalische Bedeutung vom Typ der Assoziations-Reaktion abhängt.

$$\psi_j = K_1 \times K_2 \ldots K_j = \prod_j K_j;$$

K_j: thermodynamische (experimentelle) Gleichgewichts-Konstante des j^{ten} Liganden.

$$r = \frac{B}{q}$$

Im *Scatchard*-Diagramm r/F = f(r) werden die Meßwerte aufgetragen. Die Differenzierung von Gleichung 24 ergibt folgende Grenzwerte

$$\lim_{F \to 0} = \frac{2\psi_2 - \psi_1^2}{\psi_1} \quad \text{und für} \quad \lim_{F \to \infty} = \frac{n\psi_m}{\psi_{n-1}}.$$

Der initiale Anstieg des Graphen ist also durch die Parameter der beiden ersten Bindungen charakterisiert, der finale Anstieg des Graphen ist durch die Parameter der letzten Bindung charakterisiert (30). Die Gleichung 24 ist die allgemeine Form der *Adair*-Gleichung, die 1925 entwickelt wurde, um die Bindung von O_2 an Hb zu beschreiben (n = 4)

Die *Adair*-Gleichung ist u.a. geeignet, um folgende Probleme zu behandeln:

positive Kooperativität: $\overset{1}{K} < \overset{2}{K} \ldots < \overset{n}{K}$

negative Kooperativität: $\overset{1}{K} > \overset{2}{K} > \ldots > \overset{n}{K}$

gemischte Kooperativität: $\overset{1}{K} < \overset{2}{K} \ldots \overset{i}{K} > \overset{i+1}{K} \ldots \overset{n}{K}$

gleichwertige unabhängige Bindungsstellen: $\overset{1}{K} = \overset{2}{K} = \ldots = \overset{n}{K}.$

Gleichung 24 kann durch *Sips-Hill-Nernst*-Transformation (Gl. 21) umgewandelt werden in einen Ausdruck der Art

$$\log\left(\frac{r}{1-r}\right) = \log\left(\frac{\qquad\qquad}{\qquad\qquad}\right).$$

Dieser Ausdruck geht für sehr kleine Werte von [P] über in

$$\log \overset{1}{K} + \log [P]$$

und für sehr große Werte von [P] über in

$$\log \overset{n}{K} + \log [P],$$

diese Ausdrücke definieren Asymptoten der durch Gleichung 24 definierten Funktion y = f(x). Sie ergeben (y → 0) Werte für $\overset{n}{K}$ und $\overset{1}{K}$. Diese Werte stehen in Zusammenhang mit der empirisch gefundenen Funktion (Gl. 25).

(Gl. 25) $r = \frac{K[P]^{\alpha}}{1 + K[P]^{\alpha}}$;

$$\log\left(\frac{r}{1-r}\right) = \log K + \alpha \log [P]$$

Sips-Hill-Nernst-Gleichung

α: *Sips-Hill*-Koeffizient:

$1 < \alpha \leq n$: positive Kooperation
$0 < \alpha < 1$: negative Kooperation, Heterogenität, (Quervernetzung, s. Modell 3b)
$1 = \alpha$ gleichwertige unabhängige Bindungsstellen

(Über den Zusammenhang zwischen *Adair*-Gleichung (Gl. 24) und *Hill*-Gleichung (Gl. 25) siehe l.c. (30).

Wenn (in Gleichung 24) $\overset{1}{K} = \overset{2}{K} = \ldots = \overset{n}{K}$ gesetzt wird, vereinfacht sich die Gleichung zu Gleichung 26.

(Gl. 26) $r = \frac{n\,K[P]}{1 + K[P]}$

also wird bei ^{n}Q mit n unabhängigen gleichwertigen Bindungsstellen für P pro Einheit das Ausmaß der Bindung von P n mal größer als für Q mit einer einzelnen Bindungsstelle pro Einheit mit der gleichen inneren Gleichgewichtskonstanten K.

3. Modell: m Ordnungen von Antigen-Bindungsplätzen, zu ^{m}P aggregiert.
2 Ordnungen von Antikörper-Bindungsplätzen, zu ^{2}Q aggregiert (z.B. IgG) (Modell der Präzipitation und Agglutination).

a) m = 2: Die Interaktion von zweiwertigem, im Unterschied zu ^{1}P makromolekularem ^{2}P mit zweiwertigem ^{2}Q führt, in Abhängigkeit von den Verhältnissen der Konzentrationen von ^{2}p und ^{2}q, zu linearen, gegebenenfalls zirkulären Polymeren. Unter der Annahme, daß identische und unabhängige Bindungsstellen mit intrinsischen Assoziationskonstanten $\overset{i}{K}$ vorliegen, ergeben sich die stöchiometrischen Assoziationskonstanten K aus den in Abbildung 2 zusammengestellten Faktoren (33); über entsprechende Reaktionen mit fixierten Reaktionspartnern siehe l.c. (34).

$^{1}P + {}^{2}Q$	$\rightleftarrows$	$^{1}P\,{}^{2}Q$	2	–
$^{2}P + {}^{2}Q$		$^{2}P\,{}^{2}Q$	4	M
$^{2}P + {}^{2}P_n\,{}^{2}Q_n$		$^{2}P_{n+1}\,{}^{2}Q_n$	1	M
$^{2}Q + {}^{2}P_n\,{}^{2}Q_n$		$^{2}P_n\,{}^{2}Q_{n+1}$	1	M
$^{2}P + {}^{2}P_n\,{}^{2}Q_{n+1}$		$^{2}P_{n+1}\,{}^{2}Q_{n+1}$	4	M
$^{2}Q + {}^{2}P_{n+1}\,{}^{2}Q_n$		$^{2}P_{n+1}\,{}^{2}Q_{n+1}$	4	M
$^{2}P_n\,{}^{2}Q_n$		$^{2}P_n\,{}^{2}Q_n$	1/2n	Z_n V/N

Abb. 2. Faktoren der intrinsischen Gleichgewichtskonstanten zweiwertiger Reaktionspartner (nach l.c. (33))
Erste Zeile: Reaktion des einwertigen ^{1}P
n: Anzahl der im Komplex vorliegenden Moleküle
1/2n, 1, 2, 4: Statistische Faktoren (s. Gleichung 29)
M: Korrekturfaktor für den Übergang von ^{1}P zum makromolekularen ^{2}P bei gleicher intrinsischer Assoziationskonstante $\overset{i}{K}$
Z_n: Korrekturfaktor für Ringschluß-Reaktion
V/N, (Volumen/Teilchenzahl): Korrekturfaktor für den Übergang von der bimolekularen zur monomolekularen Reaktion.

b) m > 2: Die Interaktion mehrwertiger Reaktionspartner, von denen einer mehr als zweiwertig ist, führt in Abhängigkeit von den Verhältnissen der Konzentrationen zur Ausbildung räumlich vernetzter Polymerer. Im Gleichgewicht können neben freiem ^{m}P und ^{2}Q die Komplexe $^{m}P_i\,{}^{2}Q_j$ vorliegen, mit den stöchiometrischen Indizes

$i = 1, 2, \ldots, \infty$

und

$j = (i-1),\ (i),\ (i+1),\ (\ldots),\ (i(m-1)+1).$

Die möglichen Komplexe lassen sich in einer Matrix anordnen, deren erste Zeile formal der Assoziation eines monovalenten Liganden (^{1}Q) an einen polyvalenten Binder (^{m}P) entspricht (s. Modell 2c) und deren erste Kolonne der Ausbildung linearer Polymerer durch Assoziation zweiwertiger Reaktionspartner entspricht (s. Modell 3a). Jeder dieser linearen Komplexe kann sequentiell bis zur Sättigung weiteres ^{2}Q binden, analog den in der ersten Zeile formulierten Vorgängen.

Über die Einführung der Wahrscheinlichkeitsfunktion W_P, definiert als die Wahrscheinlichkeit, daß eine Bindungsstelle auf dem ^{m}P-Aggregat mit einer

$^{m}P_1{}^{2}Q_0$	$^{m}P_1{}^{2}Q_1$	$^{m}P_1{}^{2}Q_2$	...	$^{m}P_1{}^{2}Q_m$
$^{m}P_2{}^{2}Q_1$	$^{m}P_2{}^{2}Q_2$	$^{m}P_2{}^{2}Q_3$	...	$^{m}P_2{}^{2}Q_{2m-1}$
$^{m}P_3{}^{2}Q_2$	$^{m}P_3{}^{2}Q_3$	$^{m}P_3{}^{2}Q_K$	...	$^{m}P_3{}^{2}Q_{3m-1}$

Abb. 3. Komplexe (Immunkomplexe), die durch Assoziation von m-wertigen ^{m}P und 2-wertigen ^{2}Q neben freiem ^{2}Q ($= {}^{m}P_0{}^{2}Q_1$) im Gleichgewicht vorliegen können (nach l.c. (35)).

Bindungsstelle auf dem ^{2}Q-Aggregat reagiert hat und der Wahrscheinlichkeitsfunktion W_Q, definiert als die Wahrscheinlichkeit, daß eine Bindungsstelle auf dem ^{2}Q-Aggregat mit einer Bindungsstelle auf dem ^{m}P-Aggregat reagiert hat und der intrinsischen, identischen, unabhängigen, Assoziationskonstanten $\overset{i}{K}$ lassen sich Bindungsfunktionen aufstellen, z.B. die *Scatchard*-Funktion in der Form $r' = \frac{[^{2}q] - [^{2}Q]}{[^{2}p]}$

Aus den Bindungsfunktionen läßt sich ableiten (35), daß

1. die Titrationskurve von der vorgelegten Menge ^{m}P abhängt, und daß sich die Titrationskurven bei $r' = m/2$ schneiden, wenn $\overset{i}{K}[^{2}Q] = 1/2$. Solange $[^{2}Q] < 1/2\ \overset{i}{K}$, also vor dem Schnittpunkt der Titrationskurven, ist die Bindung von polyvalenten Reaktionspartner effektiver als die von nicht-aggregierten P und Q mit gleichen intrinsischen Assoziationskonstanten. Nichtlinearität des *Scatchard*-Graphen ist beim Auftreten von quervernetzten Polymeren mit der Äquivalenz und Unabhängigkeit der Bindungsplätze vereinbar.

2. der Grenzwert $\lim_{[^{2}Q]\to\infty} r'$ wird m, das heißt unter diesen Bedingungen liegen nur noch Komplexe der Art $^{m}P_1{}^{2}Q_m$ vor. Das heißt, mit steigendem $[^{2}Q]$ wird die Konzentration aller anderen Komplexe (Abb. 3) zunächst ansteigen, ein Maximum erreichen, um dann auf 0 zurückzugehen. Alle Komplexe $^{m}P_i{}^{2}Q_j$ außer $^{m}P_1{}^{2}Q_m$ haben ihre Maximalwerte, die mit $j/i = m/2$ bei $[^{2}Q] = 1/2\ \overset{i}{K}$. Diese analytischen Ergebnisse entsprechen den Überlieferungen der empirischen Immunologie zur Deutung von Präzipitations- und Agglutinations-Reaktionen (Prozonenphänomen, Äquivalenzpunkt).

4. Modell: m Ordnungen von Antigen-Bindungsplätzen (z.B. mit $P_1 = P^*$)
n = 1 Ordnungen von Antikörper-Bindungsplätzen

(Modell zur Beschreibung des Prinzips der Bestimmung von $\overset{i}{p}$ mit Hilfe der Beziehung $[P^*Q]/[P^*] = R = f(p)$ (quantitative Bestimmung von p mit Hilfe eines der „Bindungsverfahren", z.B. Radioimmunoassay) oder auch Prüfung des Ausmaßes und der Spezifität der Bindung von P* an Q, wenn P* als „markierte" Form von P vorliegt und Q als spezifischer Rezeptor für P (z.B. P: Immundeterminante oder Hormon; Q: Antikörper oder Rezeptor)).

In diesem Modell wird in Gleichung 19

$$R_i = \frac{[P_1Q]}{[P_1]};$$

somit entsteht Gleichung 27

$$\text{(Gl. 27)}\quad R = \frac{K_1 q}{1 + K_1[P_1] + K_2[P_2]}$$

Gleichung 27 wurde nach B hin entwickelt (31), so daß sie in der Form von Gleichung 28 einer *Scatchard*-Analyse zugänglich wurde.

$$\text{(Gl. 28)}\quad K_2RB + K_2R^2/K_1 + K_1B + \\ + (1 + K_2\,(p_2 - q))\,R - K_1q = 0$$

Die Entwicklung von Gleichung 27 nach p hin wurde zur Basis der theoretischen Behandlung von Sättigungsverfahren/Kompetitionsverfahren (26, 29, 31).

$$\text{(Gl. 29)}\quad R^2 + R(1 + K_1p_1 - K_1q_1) - K_1q_1 + \\ + \frac{p_2K_2\,(R + 1)\,R}{(K_2/K_1)\,R + 1} = 0;$$

Ekins-Gleichung

Wenn mit B_0 die Konzentration von P_1Q bei $p_2 = 0$ bezeichnet wird, dann gibt es für jeden beliebigen Wert von B/B_0 zwischen 1,0 und 0,0 eine zugehörige Dosis $p_2 > 0$. Die Dosis der Substanz P_2, die zum Wert $B/B_0 = 0{,}5$ führt, wird als ID_{50} (Inhibitionsdosis 50%) der Substanz P_2 bezeichnet. Werden anstelle der bekannten Mengen der bekannten Substanz P_2 unbekannte Mengen unbekannter Substanzen (P_3, P_4 . . .) eingesetzt, so entspricht ein Vergleich der jeweiligen Dosis-Wirkungsbeziehungen dem Ver-

gleich der Dosis-Wirkungsbeziehungen zwischen einem Standard und unbekannten Proben. Parallelität der Dosis-Wirkungsbeziehung zwischen Standard und Probe ist eine notwendige, aber keine hinreichende Bedingung für die Annahme der strukturellen Identität von Standard und Probe. Diese Identität ist Voraussetzung dafür, aus der Wirkung R der Probe auf die P_2-Konzentration der Proben (P_3, P_4...) zu schließen.

Das Verhältnis äquipotenter Dosen verschiedener Substanzen, zumeist ausgedrückt als deren ID_{50}, wird als Kreuzreaktivität der betreffenden interferierenden Substanzen bezeichnet. Das Verhältnis der Kreuzreaktivitäten verschiedener Substanzen zueinander ist abhängig von den Affinitäten dieser Substanzen zu Q, d.h., von K_2, K_3, K_4 ... *und* von R, und somit von der Zusammensetzung des Systems. Wenn K_2 bekannt ist (meist geht man von der Annahme aus, daß $K_2 = K_1 = K^*$), dann kann aus dem Verhältnis der äquipotenten Mengen p_3 zu p_2 nach Gleichung 30 die Assoziationskonstante für die Substanz P_3 errechnet werden.

$$\text{(Gl. 30)} \quad K_3 = \frac{K_2}{\left(\frac{p_3}{p_2} - \frac{R}{1+R}\right)(1+R)}$$

K_2, K_3 ... haben hier die gleiche Bedeutung wie K_I in der Enzymologie. Die dort üblichen Verfahren zur Bestimmung von K_I (z.B. *Dixon*-Transformation) sind jedoch nicht übertragbar, da im allgemeinen $(P_2) \neq (p_2)$ (26).

Gleichung 30 impliziert, daß die Spezifität der analytischen Bestimmung mit Hilfe von Immunreaktionen von der Affinität, einer inhärenten Eigenschaft des Reagenz, und von den frei wählbaren Konzentrationsverhältnissen der Reaktionspartner abhängt. Dies wird auch offensichtlich, wenn man die Wahrscheinlichkeiten der Bindungen heranzieht. Die Wahrscheinlichkeit W_r einer „richtigen" Bindung (P_1Q) in Relation zu den möglichen Bindungen $(P_1Q + P_2Q + \ldots + P_mQ)$ wird durch Gleichung 31 definiert, bzw. durch W_f, die Wahrscheinlichkeit einer „falschen" Bindung $(W_r + W_f = 1)$ (36).

$$\text{(Gl. 31)} \quad W_r = \frac{[P_1Q]}{[P_1Q] + [P_2Q] + \ldots}$$

$$= \frac{[P_1]K_1}{[P_1]K_1 + [P_2]K_2 + \ldots}$$

$$= \frac{(p_1 - [P_1Q])K_1}{(p_1 - [P_1Q])K_1 + (p_2 - [P_2Q]K_2 + \ldots}$$

1. Wenn $p_1 + p_2 + \ldots \gg q$, dann $p_1 - (P_1Q) \approx p_1$
$p_2 - (P_2Q) \approx p_2$

$$W_r = \frac{p_1K_1}{p_1K_1 + p_2K_2 + \ldots} = 1 + \frac{p_1K_1}{p_2K_2} + \ldots$$

$$W_r - 1 = -W_f = \frac{p_1}{p_2}\frac{K_1}{K_2} + \ldots \text{ oder,}$$

$$\text{da } K = e^{-\frac{\Delta G^\circ}{RT}}$$

$$W_f = -\frac{p_1}{p_2} e^{-\frac{(\Delta G_1^\circ - \Delta G_2^\circ)}{RT}}$$

Also ist die Wahrscheinlichkeit des Auftretens von (P_1Q) bei Einsetzen äquimolarer Mengen von p_1 und p_2 $\left(\frac{p_1}{p_2} \approx 1\right)$ von der Relation der Assoziationskonstanten bzw. von der Differenz der *Gibbs*schen Potentiale abhängig. Bei realen Ansätzen wird die apparente Wahrscheinlichkeit durch das Verhältnis p_1/p_2 beeinflußt.

2. Wenn $p_1 + p_2 + \ldots \ll q$, dann wird $[P_1Q] \approx p_1$
$[P_2Q] \approx p_2$

Somit wird W_r unabhängig von K_1/K_2 und das Auftreten der Komplexe P_1Q und P_2Q wird proportional den eingesetzten Mengen von p_1 und p_2, sofern K_1, K_2 Mindestwerte überschreiten.

Um hochspezifische Reaktionsergebnisse zu erzielen, ist es also erforderlich, daß die Differenzen der *Gibbs*schen Reaktionspotentiale der Assoziationen von Q mit dem „richtigen" Liganden P_1 und dem „falschen" Liganden P_2 groß genug sind, und daß $p_1 < q$.

4. Anwendung physikalisch-chemischer Kenngrößen in der analytischen Praxis

Wenn die physikalisch-chemischen Kenngrößen der Immunreaktion bekannt sind, können mit Hilfe der Reaktions-Geschwindigkeits- und Gleichgewichts-Konstanten Formeln entwickelt werden, nach denen die Meßdaten der nach „Bindungsverfahren" durchgeführten quantitativen Bestimmungen ausgewertet werden können („Standardkurven"). Derartige Auswertungsverfahren erweisen sich dann als vorteilhaft gegenüber phänomenologischen Dosis-Wirkungs-Kurven, die ausschließlich über statistische

Interpolations- und Regressionsverfahren gewonnen werden (deren Algorithmus keinen funktionalen Zusammenhang mit Reaktionsmechanismen besitzt, wie z.B. polygonale Interpolation, Spline Approximation), wenn die Modellvorstellungen über die Reaktion weitgehend den realen Reaktionsbedingungen entsprechen. Tatsächlich konnten bewährte Auswertungsverfahren, die zunächst empirisch gewonnen worden waren, schließlich auf die Thermodynamik der Reaktion zurückgeführt werden (37). Analoge Überlegungen gelten für die Optimierung der Reaktanden-Konzentrationen und Reaktionsbedingungen. Auf der Basis der Reaktionsgleichungen und der Fehleranalysen können Strategien zur Optimierung entwickelt werden, womit zumeist eine Maximierung der Empfindlichkeit einer Bestimmung gemeint ist; auf die Möglichkeit der Maximierung der Spezifität durch Berücksichtigung thermodynamischer Parameter wurde bereits hingewiesen (s. 4. Modell). Die Praktikabilität, sofern damit die Robustheit der Bestimmung und ihr Zeitaufwand gemeint ist, wird ebenfalls durch thermodynamische Daten determiniert. Reaktionsabbrüche vor Erreichen des Gleichgewichtszustandes (z.B. beim Sequentialverfahren) führen im allgemeinen zu Meßwerten, die stärker mit Fehlern behaftet sind, als wenn Gleichgewichts-Konzentrationen gemessen werden. Schließlich ist die Kinetik und damit auch die Enthalpie der Reaktion, und zwar sowohl die der Reaktion mit dem Analyten wie auch die der Reaktion mit anderen Kompetitoren des Indikators, ausschlaggebend dafür, wie weit die Inkubationszeit abgekürzt werden kann und wie weit bei höheren Temperaturen inkubiert werden kann, ohne daß unvertretbare Einbußen an Empfindlichkeit und Spezifität die Folge sind (26, 38–42). Eine Optimierung der „Bindungsverfahren" durch „trial and error" ist zwar auch möglich und letztlich unvermeidbar, aber ohne Kenntnis der thermodynamischen und reaktionskinetischen Zusammenhänge nicht planvoll einzusetzen.

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Polyclonal and Monoclonal Antibodies as Reagents in Biochemical and in Clinical-Chemical Analysis[1])

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Summary: Conventionally prepared polyclonal antibodies have been used for a long time in biomedical research and in clinical-chemical diagnosis. The hybridoma technology introduced by *Köhler & Milstein* ((1975) Nature *256*, 495–497), has opened the way to a new dimension in serology. It is now possible to prepare monoclonal antibodies to any determinant on any component of biological matter. Such monoclonal and thus specific antibodies can be obtained even against previously unknown antigens which had not been available in purified or enriched form. Thus the dreams of immunologists and clinical scientists who were searching for new disease-related markers, have become a reality.

The present impact and the predictable future influence of these new developments on biomedical research and especially on clinical-chemical diagnosis, as well as their potential, limitations and problems, will be critically reviewed in this paper. As an example of the potential of the new technology, recent results of tests on the quantitation of urinary kidney-derived antigens with the help of monoclonal antibodies are presented. With these monoclonal antibodies, which are specific for antigens in defined regions of the nephron of the human kidney, recognition of the location and extent of primary damage at the cellular level will be possible without invasive techniques.

Polyklonale und monoklonale Antikörper als Reagenzien in der biochemischen und klinisch-chemischen Analytik

Zusammenfassung: Konventionell hergestellte polyklonale Antikörper haben schon seit langer Zeit ihren festen Platz als Reagenzien in der biomedizinischen Forschung und in der klinisch-chemischen Diagnostik. Die Hybridomtechnologie mit der sich ergebenden Möglichkeit zur Herstellung monoklonaler Antikörper gegen jede Determinante jedes denkbaren Antigenmoleküls hat das Spektrum der Anwendungsmöglichkeiten serologischer Techniken in unvorstellbarer Weise erweitert. So ist es jetzt möglich geworden, monoklonale und damit spezifische Antikörper auch gegen Determinanten bisher unbekannter Antigene zu gewinnen, ohne das entsprechende Antigenmolekül gereinigt oder angereichert zu haben. Die Wunschträume der Immunologen und klinischen Forscher, die nach neuen diagnostischen Markern, vor allem in der Tumorforschung, suchten, wurden greifbare Wirklichkeit.

Die vorliegende Veröffentlichung soll diese neuen Entwicklungen mit ihren – bereits heute vorhandenen und in Zukunft absehbaren – Auswirkungen auf die biomedizinische Grundlagenforschung, vor allem aber auf die klinische Diagnostik darstellen und ihre Möglichkeiten, Grenzen und Probleme kritisch diskutieren. Die Möglichkeiten werden exemplarisch am Beispiel von Tests gegen Antigene der menschlichen Niere gezeigt. In diesen Tests werden monoklonale Antikörper gegen Antigene aus genau lokalisierten Bereichen des Nephrons eingesetzt, um die entsprechenden Antigene bei pathologischen Veränderungen im Harn von Patienten nachzuweisen. Solche Tests eröffnen die Möglichkeit zur Erkennung von Lokalisation und Ausmaß einer Schädigung im Nierenparenchym ohne die Anwendung invasiver Techniken.

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

Introduction

Substances defined by their immunological rather than by their enzymatic activity, and therefore designated "antigens", have played a minor role in clinical chemistry and diagnosis. The sprectrum of tests available for such antigens has been very limited and still is today. This is mainly due to technical problems in the development of tests for marker substances without known biological activities.

In the first seven decades of this century, basic biochemical research concentrated on substances with known biological activities such as enzymes and hormones. Most of the enzyme tests in use today in biomedical research and in clinical diagnosis were known from the outset. With such tests, it was relatively easy to identify organ or tissue distribution of a large number of marker substances and to determine their concentration in body fluids under normal and pathological conditions.

The development of tests for proteins or other cellular components without known biological activities has been hampered by the fact that these substances do not exhibit any known measurable biological function. It is, however, possible to characterize these, as well as any other biological marker, by their reaction with specific antibodies. Preparation of specific antibodies depends on the availability of the corresponding pure antigens. For the purification of antigens recognition systems are required. Tests based on specific antibodies could serve as such recognition systems. Since, however, specific antibodies require pure antigens and, since the preparation of pure antigens requires specific antibodies, we find ourselves lost in a vicious circle.

In a few cases it has been possible to prepare specific antibodies against antigens not known before and not available in pure form; α-foetoprotein is an example for such an antigen. Furthermore, specific antibodies have been prepared against constituents of human serum rather early in the history of clinical chemistry. These antigens were, however, so "prominent" that no special recognition systems were required to identify them.

In principle, it should be possible to prepare specific antisera for any known antigen in a way similar to the procedure used for the preparation of antibodies to α-foetoprotein: after immunization of a laboratory animal with the serum of a patient suffering from a defined disease and absorption of the antibodies to normal serum components with normal human serum, an antiserum specific for those markers that are released under this specific pathological condition should be obtained.

In reality, however, this is impossible, since in contrast to α-foetoprotein, which is present in the sera of foetuses and patients with liver carcinoma in considerable concentration, most of the marker antigens of interest are present only in minute concentrations, in the range of 1 μg/l to 1 mg/l, as we know from clinical enzymology. To raise specific antibodies against such "minor contaminants" in the serum is almost always impossible. The laboratory animal's immune system confronted with hundreds or even thousands of different antigens most of them in very low concentrations, remains unresponsive to most of them.

Nevertheless, we know that large numbers of such antigens must be present in the sera of patients, released from cells as the result of pathological alterations or lesions. The exploitation of this vast pool of diagnostically valuable molecules has recently become possible by the development of a new technology for the preparation of *monoclonal* antibodies. The hybridoma technology, developed by *Köhler & Milstein* (1) in 1975 has opened the way to the preparation of *monoclonal antibodies with predefined specificities* against any determinant of any antigen of biological matter. Such reagents have been the dream of immunologists. Therefore, many research groups have concentrated their efforts on the preparation of such monoclonal antibodies to a variety of antigens in many fields of biomedical research. In this paper, the advantages of monoclonal antibodies, their future applications in various fields of research, and, last but not least, their limitations will be discussed. The use of monoclonal antibodies for the development of new strategies in kidney diagnosis, using urine, will be discussed as an example of these future developments.

The Polyclonal Antibody Response

Conventionally prepared antibodies have been used for many decades in biology and medicine for research as well as for diagnostic and therapeutic purposes. Although they have many uses, their application has been limited by their inherent biochemical properties. During its development, the immune system was exposed to an uncountable number of challenges from its antigenic environment and has acquired, as a result, the ability to respond with an equally complex diversity of antibodies. Even against a very simple chemically homogeneous de-

terminant like the dinitrophenyl group, artificially introduced into a carrier molecule, a population of antibodies with different affinities and fine specificities is induced.

Immunization of an animal with a substance foreign to its immune system – therefore called "immunogen" or "antigen" – produces a very specific polyclonal cellular and humoral response. Individual cells of the immune system recognize antigenic "determinants" or "epitopes", individual molecular structures on the surface of the antigenic particle. Consequently, they are stimulated to produce large numbers of antibodies, protein molecules complementary to the corresponding epitopes. Since each antigenic particle exposes an undeterminable number of individual epitopes on its surface, this stimulation will result in an undeterminable number of individual antibodies, a polyclonal antibody population. This antibody population is found in the serum of the immunized animals.

Each antibody has a binding site, a molecular structure by which it can recognize the complementary structure on the antigen particle, the epitope, and bind to it. Binding of antibodies to an antigen can have several consequences: inhibition of biological activities of the antigen (toxin, enzyme, bacteria, virus); complex formation (precipitation, agglutination); removal or destruction of the antigen by accessory biological systems (macrophages, complement).

Since the animal's immune system will be stimulated in an unpredictable way by each of the epitopes present on each of the antigens and, in addition, by each contamination in the antigen preparation used for immunization, high purity of the antigen is one of the most important preconditions for the preparation of specific antisera. Very often it is difficult or impossible to obtain highly purified antigens. Sometimes, as is the case with membrane integrated antigens, purification of the antigen is not possible without destruction of its native structure. Preparation of antisera specific for subunits of complex biological molecules like enzymes is equally difficult since the native biological structure is often preserved only in the integrated native molecule. The preparation of antibodies to previously unknown biological molecules was not possible at all through conventional immunization procedures.

The Hybridoma Technology

The hybridoma technology has opened a new dimension in the field of immunology and provided a way of solving some of the problems inherent in polyclonal antisera. Through in vitro fusion of sensitized B-lymphocytes, the precursors of antibody producing plasma cells, with "immortal" plasmacytoma cells, i.e. continuously dividing transformed plasma cells, it became possible to dissect the animal's polyclonal immune response into its monoclonal components and thus to prepare monoclonal antibodies.

The technological inventions necessary for the development of the hybridoma technology had been made a long time previously. Continuously dividing immortal plasmacytoma cell lines of the mouse were first developed by *Potter* (2), adapted to tissue culture conditions in 1970 by *Horibata* (3) and further developed by others (4) including *Köhler, Howe & Milstein* (5).

For selection of fused from unfused cells, mutant plasmacytoma cell lines had to be developed that are deficient in an enzyme present in normal mouse cells. A mutant deficient in the salvage pathway enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) was developed by *Cotton* et al. (6) and by *Köhler* et al. (5). Hypoxanthine-guanine phosphoribosyl transferase, a membrane bound enzyme, allows the cell to take up hypoxanthine, a nucleotide precursor, from the culture medium through transmembrane transport and convert it into the nucleotides needed for DNA synthesis (7). In general, this pathway is not important for the cell's survival because nucleotides can equally well be acquired by de novo synthesis. If, however, the DNA de novo synthesis pathway is blocked, the cells are bound to make use of the external nucleotide precursor hypoxanthine. In the hypoxanthine-aminopterine-thymidine (HAT) selection medium developed by *Littlefield* in 1964 (8), the de novo synthesis pathway is blocked by aminopterine and only cells with an intact hypoxanthine-guanine phosphoribosyl transferase salvage pathway enzyme can survive. Unfused hypoxanthine-guanine phosphoribosyl transferase deficient plasmacytoma cells, although immortal in principle, cannot survive under these conditions. Unfused lymphocytes, mortal in vitro, will only survive a few days although their hypoxanthine-guanine phosphoribosyl transferase enzyme is fully active. Only hybrids between the hypoxanthine-guanine phosphoribosyl transferase deficient plasmacytoma cells and normal mouse lymphocytes will survive in vitro. Thus, by means of fusion, two genetic factors can be combined in one cell: the immortality and capability for unlimited growth of the plasmacytoma cell and the capacity to produce one individual monoclonal antibody of the plasma cell.

Originally *Köhler & Milstein* used Sendai virus as the fusing agent (1). Later, however, polyethyleneglycol (PEG), a fusiogen found by plant cytologists, was adapted for fusion because of its higher fusion rate and easier application (9, 10). In practice, fusion is done by mixing, in the presence of the fusing agent, the spleen lymphocytes of an immunized mouse with mouse plasmacytoma cells harvested from in vitro culture. Fusion takes place randomly within a few minutes. After removal of the fusing agent the cells are distributed into the wells of culture plates and fed with HAT selective medium. After 1—2 weeks unfused plasmacytoma and spleen cells as well as inter-plasmacytoma and inter-lymphocyte fusion products will have died, and rapid cell growth will be observed only in those wells that contained at least one hybrid cell in the cell mixture seeded after fusion. After 2—3 weeks the selective medium is replaced by normal culture medium and the cell samples can be further processed. The rapidly growing hybrid cells, called hybridomas, secrete monoclonal antibodies into the culture fluid. Their specificities can be tested by suitable tests.

Details of the fusion protocols and mouse myeloma cell lines available for fusion have been published in various technical papers (11—15). The principle is depicted in figure 1.

Immunization procedures vary greatly from laboratory to laboratory. In our experience, a long term hyperimmunization procedure with up to 12 injections of the antigen mixture in complete *Freund*'s adjuvent has led to promising results for the production of monoclonal antibodies to epitopes of tissue components so far unknown.

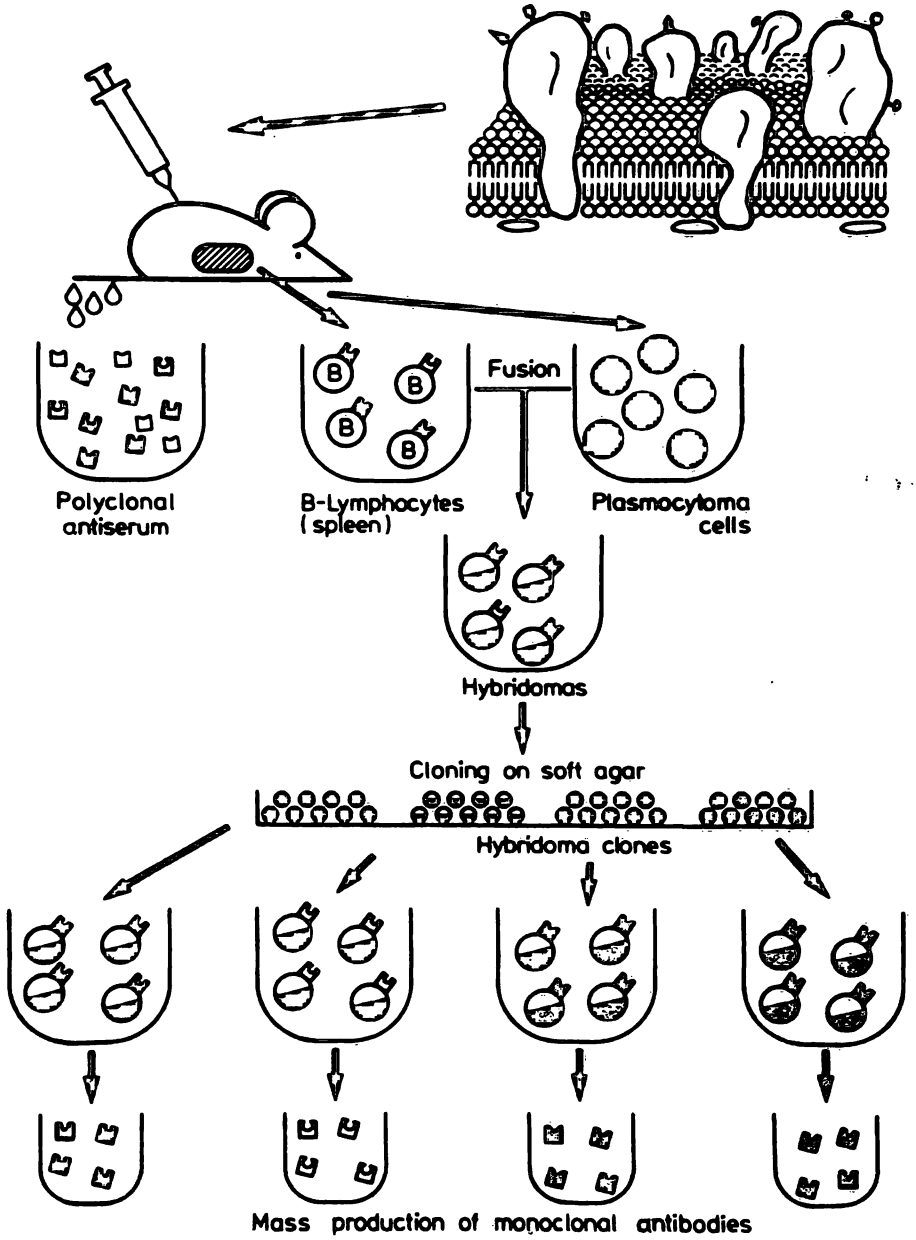

Fig. 1. Schematic presentation of preparation of monoclonal antibodies to membrane antigens. Taken from: *Falkenberg* et al. (68).

Production and Purification of Monoclonal Antibodies

Monoclonal antibodies excreted into tissue culture fluid by hybridoma cells can be detected by suitable techniques. In most cases, antibody concentration in culture fluid is high enough (10—50 mg/l) for routine tests such as RIA, ELISA, indirect immunofluorescence, staining with peroxidase-labelled antibodies, autoradiography. Those hybrid cell samples that secrete a monoclonal antibody of desired specificity have to be checked for monoclonality. The probability of monoclonality depends on the set-up of the experiment and especially on the frequency of wells with cell growth and can be calculated accordingly (16, 17). However, in most cases, cloning will be necessary, either by limiting dilution or on soft agar. Mass production of monoclonal antibodies is then possible either from cell culture supernatant or from ascitic fluid of hybridoma tumour bearing mice. For purification of monoclonal antibodies several methods can be applied:

- affinity chromatography on protein A columns (18) (applicable only to monoclonal antibodies of some IgG-subclasses)
- immunospecific affinity chromatography on antigen columns or on antimouse-Ig-columns: Careful selection of the dissociating agent is important to prevent irreversible damage to the antibody molecules
- conventional biochemical techniques such as ion exchange and gel permeation chromatography. The HPLC technique has recently been applied successfully to monoclonal antibody purification (19, 20).

Problems with Monoclonal Antibodies

However, it has turned out to be rather difficult to obtain monoclonal antibodies of high purity. Monoclonal antibodies in ascitic fluid are contaminated with normal mouse serum proteins, including normal mouse IgG's which cannot always be removed by the purification procedures mentioned above. Monoclonal antibodies in tissue culture fluid are free of other mouse proteins. They are, however, heavily contaminated with proteins from horse serum or from foetal calf serum (e.g. 10 µg of monoclonal antibodies in 5000 µg of horse serum proteins).

The affinity and specificity of monoclonal antibodies are not always adequate. Since each monoclonal antibody is just one of the total population of antibodies elicited against each of the epitopes of a multiepitope-antigen, it might not show the desired epitope specificity and affinity which makes it suitable for a certain test (e.g. RIA, ELISA, Immunofluorescence, etc.). Recently (21, 22) it was reported that monoclonal antibodies might exhibit a kind of "assay-specificity", indicating that a given monoclonal antibody might perform excellently in one assay but give poor or negative results in another. Therefore, large screening programs have to be performed to identify those monoclonal antibodies that exhibit the desired specificity and sufficient affinity for their antigen(s).

In our experience, there is no problem in testing monoclonal antibodies, even those of low affinity, with techniques in which one of the reaction partners is fixed to a solid phase (e.g. solid phase RIA, ELISA, Immunofluorescence, Immunoperoxidase).

Further problems might arise from the "cross-reactivity" of monoclonal antibodies (23). The binding site of a monoclonal antibody is complementary to a three dimensional molecular structure on the antigen molecule, the epitope. On protein molecules such structures might be determined by the primary sequence of a few amino acids or by a steric configuration of several closely or distantly located amino acid residues. Since the molecular area forming such epitopes is rather small, the same or similarly constructed molecular areas might be present on other related or non-related proteins. This "blurred vision" of the monoclonal antibodies causes considerable problems for their use. Although these considerations are also valid for each of the antibodies that form a polyclonal antiserum, these problems are not encountered because all the antibodies of the serum react with *one* target antigen for which a high specificity is thus obtained. It might be necessary in the future to combine several monoclonal antibodies to form artificial antisera-like oligoclonal mixtures.

Due to the individual structure in the constant (class and subclass specific sequences) and variable (idiotypic sequences) regions each monoclonal antibody is a distinct individuum and has to be treated as such. Techniques and procedures developed for the biochemical and immunochemical manipulation of antibodies from conventionally prepared polyclonal antisera cannot always be applied to these "capricious primadonnas" of monoclonal antibodies. Digestion with proteases to produce Fab-fragments or conjugation with marker molecules do not always work as expected and cause problems in the preparation of derivatives of monoclonal antibodies (24).

Application of Monoclonal Antibodies Instead of Conventional Sera

Monoclonal antibodies have many advantages over conventional polyclonal antisera. Nearly everything that has been done with antisera might be done better with monoclonal antibodies. The most important advantages and disadvantages of working with monoclonal antibodies are summarized below:

Advantages

- No pure antigen needed: total cells, unfractionated cell extracts or partially enriched fractions can be used
- Purification of antigens on immunosorbent columns possible
- Unlimited supply of each monoclonal antibody
- High reproducibility over unlimited periods of time
- Worldwide standardization possible for the first time
- Useful for all kinds of binding techniques, especially in solid phase systems
- Localization of individual membrane determinants on the cell (topography)
- during cell cycle and cell differentiation
- in relation to other membrane antigens
- Localization of individual determinants of an antigen (external or internal part of the antigen)
- Determination of the shed (physiological condition) or solubilized (pathological condition) antigen or of antigen fragments in extracellular fluids

Disadvantages

- Affinity of monoclonal antibodies is often low
- Precipitation often not possible
- Expansion of work
- High costs

Scientists around the world have prepared monoclonal antibodies against nearly every antigen one can think of. The information collected is immense and growing day by day.

Industry has followed the trend. Many tests, previously performed with conventional antisera, are now on the market using monoclonal antibodies. Predictions from the economic sector point to the development of a 2 billion dollar industry world wide by 1990 (25). At this time a 50% replacement of polyclonal antibodies by monoclonal antibodies in diagnostic tests is forecasted (26).

In the first instance, development was aimed at replacing polyclonal antisera by monoclonal antibodies. By this approach new and better reagents for research and clinical-analytical purposes were developed. However, the high costs of the technique and the unavoidable large screening programmes resulted in sales prices often prohibitive for the reduced budget of the university research worker. In many cases it is questionable whether a cheap polyclonal antiserum should be replaced by an expensive monoclonal antibody.

There are, however, problems in which monoclonal antibodies due to their specificity and biochemical homogeneity have definite advantages over polyclonal antisera. Since monoclonal antibodies are specific for individual antigenic epitopes, tests for haptens and peptide hormones built on monoclonal antibodies might be better and more specific than conventional tests applying absorbed polyclonal antisera. If a hybridoma producing an antibody with the desired properties has been found, production of the monoclonal antibody is always simpler and cheaper than the production of polyclonal antisera which each have to be rendered specific by tedious absorption procedures. Hormones that exhibit partial structural identities or homologies in their peptide chains, e.g. thyrotropin, follitropin, human chorionic gonadotropin and lutropin, are examples of this approach. In tests for the quantitation of such hormones in body fluids monoclonal antibodies will compete with absorbed polyclonal antisera on the market.

For the determination of haptens like digoxin and structurally related compounds and breakdown products thereof, monoclonal antibodies might be better than polyclonal antisera (27). In this case, high affinity of the antibodies is, however, of greatest importance for the development of suitable tests. Although the search for a monoclonal antibody that fulfils all the conditions might be long and tedious, the chances of finding it are better than the chances of finding a polyclonal antiserum with the desired properties.

The evaluation of isoenzymes, microheterogeneous forms and genetic variants of enzymes by immunological techniques is another field of application of monoclonal antibodies. Polyclonal antisera might be as good or better than monoclonal antibodies as was shown with the creatine kinase MB isoenzyme. Inhi-

bition obtained with monoclonal antibodies (28) to the B subunit was nearly as high as with polyclonal antibodies.

There are other isoenzymes which so far can be recognized only by electrophoretic techniques. Since the difference in electrophoretic mobility must have a structural basis, it should be possible to prepare monoclonal antibodies with the power to identify individual isoenzymes. The amylase isoenzymes are examples of this kind. Differentiation between the pancreas-type and the saliva-type isoenzyme is possible by electrophoretic mobility, not by polyclonal antisera (29). In this case monoclonal antibodies will be useful reagents.

Several groups have prepared monoclonal antibodies against prostatic acid phosphatase (PAP). Monoclonal antibodies seem to have advantages over polyclonal antisera in test systems, since some of the monoclonal antibodies do not cross-react with non-prostatic acid phosphatases (30). A "tandem" assay using two monoclonal antibodies specific for two epitopes of the prostatic acid phosphatase molecule was found to be specific exclusively for the prostatic enzyme and useful for prostatic acid phosphatase measurement (31). The properties of monoclonal versus polyclonal antibodies to prostatic acid phosphatase in histochemical staining are compared in a recent publication by *Shevchuk* et al. (32). Most carcinomas were positive with a goat antiserum, fewer with a rabbit antiserum, and only about 50% were positive with the monoclonal antibodies. This might reflect the heterogeneity of the antigens used for preparation of the antisera. For clinical testing, a broad spectrum antiserum might be most useful. For research purposes, however, where absolute specificity is needed to gain new information, monoclonal antibodies may be more useful.

In test systems developed for carcinoembryonic antigen (CEA), two site-directed monoclonal antibodies are used. Since "nonspecific cross-reacting antigens 1 and 2 (NCA-1 and NCA-2)" are not recognized, such a test system based on monoclonal antibodies offers advantages over conventional test systems (33). The sensitivity and specificity of 3 different forms of solid phase EIA using 5 different monoclonal antibodies against 4 distinct epitopes of the CEA molecule were compared by *Buchegger* et al. (34). In two further recent publications, the detection of "epitope heterogeneity" of CEA from various origins was reported. *Eshhar* et al. (35) found that a series of 50 monoclonal anti-CEA antibodies could be divided into four groups according to their reactivity with individual epitopes in CEA preparations from tissues and tumours of various origins. Similar observations were made by *Wagner* et al. (36) who found that 5 monoclonal antibodies directed against the protein moiety of the CEA molecule were helpful in differentiating between 5 epitopes on CEA and/or different CEA related antigens in normal and malignant tissues.

"Silent" enzymes, i.e. inactive (e.g. proteases) or inactivated forms or breakdown products of biologically active molecules are another field of application of monoclonal antibodies. Monoclonal antibodies to phenylalanine hydroxylase have been prepared in our laboratory (37) in order to differentiate between various genetic variants of the enzyme and thus provide a means of classifying the hereditary disease phenylketonuria.

Modulation of enzyme activity by antibodies is a well known phenomenon. With monoclonal antibodies, it is now possible to draw conclusions concerning the molecular site involved in enzyme activity regulation and modulation by antibodies. The activation of a defective enzyme, β-galactosidase of *E. coli* carrying Z-gene point mutations, by individual monoclonal antibodies has been reported recently (38) and indicates that a "single hit" modification of protein conformation is possible. In our University, *Hessova* et al. (39) have prepared monoclonal antibodies to the oligomeric enzyme phosphorylase kinase which allows characterization of the function of the α- and β subunits. Whereas most of the monoclonal antibodies inhibited the A_1 or the A_2 or both activities, some were shown to activate the A_0 or the A_1 activities. Informative relationships were observed between the binding to a specific subunit and the enzyme activity.

Monoclonal antibodies to markers of classes and subclasses of human and murine immunoglobulins have already become important tools in research, especially in hybridoma research, as well as in clinical diagnosis. Problems arising from unexpected and so far unexplained reactions and cross-reactions (21, 22) of monoclonal antibodies specific for immunoglobulins of the IgG subclasses are being investigated in a collaborative study, organized under the auspices of the IUIS and the WHO. During the 6th European Immunology Meeting in Interlaken in September 1984, the colloquium on "Monoclonal antibodies to human Immunoglobulins – Specificity and application" is devoted to this question.

A whole generation of new test systems has become possible by the introduction of monoclonal antibodies: "enzyme channeling" (40) or "proximal linkage" (41) immunometric assays in which a measur-

able substrate turnover is observed *only when two* monoclonal antibodies, each specific for one individual epitope of the same particle, and each labelled with another enzyme react with the antigen. The "sandwich ELISA" with two monoclonal antibodies directed against two different epitopes of the antigen, one bound to the solid phase ("catcher") the other labelled with a marker enzyme ("indicator") can be done in a "one step" mode: the antigen-containing specimen *and* the enzyme labelled monoclonal "indicator" antibody can be added to the solid phase bound "catcher" in a mixture. This saves time and costs in the clinical laboratory.

In this context a paper published 1980 by *Kohen* et al. (42) should be mentioned. In this paper the authors reported that a monoclonal antibody directed against the dinitrophenyl (DNP) hapten exhibited quasi-enzymatic properties if the DNP-group was conjugated covalently through an ester bond with the fluorescent compound 7-hydroxy-coumarin (DNP-CU). The antibody-enhanced esterolysis of DNP-CU results from the interaction of the labile ester with a nucleophilic group in a proper position within the antibody combining site.

Exploitation of the Advantage of the Hybridoma Technology: Tissue Antigens

Most of these applications of monoclonal antibodies are not of great interest to immunologists, since the new technology is only used *instead* of the conventional forms of antibody production, exploiting only part of the manifold advantages of the new technology. In these developments, however, the usefulness of the new technology has been tested and the question answered whether the advantages compensate for the enormous costs of development.

In order to exploit all the possibilities of the hybridoma technology, new approaches and strategies had to be devised. With monoclonal antibodies it is possible to detect new antigens or antigenic determinants which, for technical reasons, have not been accessible before. Monoclonal antibodies can be raised against any determinant of a cell, a membrane fraction, or a tissue extract. In very tedious and expensive screening procedures, those have to be selected that are specific for disease-relevant tissue marker antigens. With these antibodies, the purification and characterization of the corresponding antigens are then possible.

Monoclonal Antibodies to Lymphocyte Differentiation Antigens

One of the most important developments in this field concerns the recognition of subsets of the lymphoid cell population in human blood (43). A series of monoclonal antibodies has been prepared, each directed against a differentiation antigen by which a certain T-cell type is characterized. Thus, the determination of total T-cell, T-suppressor- and T-helper cell counts in blood has now become possible. Monoclonal antibodies together with the new fluorescence-activated cell sorting and counting technique (44) will in future enable the haematologists to obtain information on the cellular immune status of a patient. Such diagnostic tools might in future enable the clinician to draw conclusions on the state of a disease and help him to decide on appropriate therapeutic measures. In urology these new reagents already have diagnostic as well as therapeutic consequences, e.g. in kidney transplantation. Determination of helper to suppressor T-cell ratio might help to predict the survival of a transplant in a recipient (45, 46). The successful treatment of rejection episodes in kidney transplant recipients with monoclonal anti-T-cell antibodies has been reported recently (47). *Cosimi* et al. (48) treated cadaver donor renal allograft recipients at the time of diagnosis of acute rejection with monoclonal OKT3 antibody (reactive with all mature peripheral blood T-cells). In all 8 cases loss of essentially all detectable peripheral blood OKT3-reactive cells was observed within 5 minutes of i.v. infusion of 1—5 mg of OKT3 antibody, and the established rejection episodes were reversed in all cases within 2 to 7 days without any treatment other than OKT3 antibody. *Jonker* et al. (49) used a rhesus monkey skin allograft model to study the effects of administration of monoclonal antibodies in detail. They found that the monoclonal OKT4 and OKT4A antibodies specific for T-helper-cells successfully prolonged allograft survival whereas the monoclonal OKT8 antibody specific for cytotoxic/suppressor T cells did not.

It can be expected that monoclonal antibodies against clearly defined T-cell-subpopulations will replace the previously used horse anti-human-thymocyte globulin (ATG) with its unselective T cell killing capacity (50).

Since malignant lymphocytes express normal as well as disease-related antigens on their surface, the characterization of leukaemic cells, the categorization of leukaemias, and, finally, new therapeutic approaches have become possible with monoclonal antibodies (51).

Currently, a simple test for the quantitation of T-cell marker antigens on peripheral lymphocytes is under development at Boehringer Mannheim (52): In this test, which can be performed in a normal clinical laboratory following routine procedures, T-lymphocyte antigens are determined with galactosidase labelled monoclonal antibodies using the lymphocytes themselves as solid phase. By this test, the determination of T-cell subset patterns which previously was possible only in laboratories equipped with highly sophisticated machinery (fluorescence activated cell sorter, FACS) or in tedious manual cell counting under the fluorescence microscope, becomes available to every laboratory which is equipped with a centrifuge and a photometer.

Monoclonal Antibodies to Tumour Antigens

The ability to prepare monoclonal antibodies to previously unknown biological marker molecules initially raised many hopes in the field of tumour immunology. Monoclonal antibodies, directed to tumour-specific or tumour-associated marker molecules, were thought to offer the ultimate solution to the cancer problem. The main obstacle to this approach lies in the animal's undifferentiated immune response to everything that is foreign in the sample used for immunization. Thus immunization with human tumour cells, tumour cell extracts, or fractions thereof will yield, after fusion, monoclonal antibodies directed against whatever is recognized as foreign by the animal's immune system. Considering the vast complexity of tissue antigens, monoclonal antibodies to real tumour antigens can be found only by chance.

As discussed earlier, monoclonal antibodies to well known tumour markers like carcinoembryonic antigen (CEA) or prostatic acid phosphatase (PAP) seem to offer advantages over conventionally prepared antisera. The literature on new tumour marker antigens detected by monoclonal antibodies is growing day by day. Most of these reports have, however, to be taken with great reservation. So far, none of the new markers has become part of clinical diagnostic routine procedures. It is hoped that the efforts undertaken in many research laboratories around the world will lead to the development of new diagnostic tools that will allow early detection and better prognosis of cancer diseases. In several papers (53–57) and monographs (58, 59) the new developments have been described in detail and critically reviewed. The future prospects of the use of targeted monoclonal antibodies for radioimmunoimaging (RII (60, 61)) and for radioimmunotherapy (RIT (61, 62)) are equally promising. *Paul Ehrlich*'s view (63) of targeted antibodies used as "Zauberkugeln" (magic bullets) or, in modern terms as "guided missiles" against cancer might become reality in the near future.

Monoclonal Antibodies to Kidney Tissue Antigens

Diagnosis of kidney damage is generally obtained from the results of tests of renal glomerular or tubular function. Changes in function, however, indicate the existence of severe lesions or irreversible tissue necrosis. Thus, function parameters allow recognition of *late phase* reactions occurring during kidney injury but they are in most cases inadequate for the determination of minor *early phase* alterations. The latter can be reversible and disappear when the aggression on the tissue ends. They consist of modifications at the level of the renal cell surface accompanied by release of membrane components in the urine. Such early phase lesions can sometimes be recognized by electron microscopic examination of biopsy specimens. Most of the tests performed on urine in use today yield information on late phase events only. The recent establishment of assays for quantification of the urinary β2-microglobulin (64) and for measurement of the urinary alanine aminopeptidase activity (65) has allowed recognition of early phase alterations. The determination of other kidney-derived components in urine may in addition facilitate the localization of damage to particular sites of the nephron. In order to search for kidney-derived components in urine we have prepared monoclonal antibodies against human kidney antigens (66, 67). These antibodies display various specificities for antigens in the different parts of the nephron, in blood vessels and in the interstitia.

Initial evidence for the presence of kidney-derived antigens in urine was obtained by "inhibition of immunofluorescence staining" assays performed with concentrated urine of patients suffering from various kidney diseases (68). In order to detect and quantify antigens in native urine, more sensitive tests had to developed. The sandwich ELISA was chosen because it allows estimation of antigens which are not available in pure form as is the case with these kidney-derived antigens.

A number of sandwich ELISA tests have been developed by using various combinations between solid phase adsorbed "catcher" and glucose oxidase conjugated "indicator" antibodies. Native urine samples of patients suffering from various kinds of kidney

damage were assayed with each of these test systems, and the percentage of urines containing antigens were calculated. Thus the sandwich ELISA, a very quick and easy technique, appeared to be suitable for the detection of antigens in native, i.e. unconcentrated and undialysed, urine.

Each of the monoclonal antibodies used is specific for one of the various epitopes of the kidney-derived urinary antigens. Some of these epitopes can be situated on the same antigen. Consequently, it is possible that some of the sandwich ELISA tests measure the same antigen(s), although each antigen was bound to the various "catcher" antibodies by means of a different antigenic epitope. In order to test this hypothesis and to select those assays that allow detection of different urinary antigens, correlation analysis (*Spearman*) of the data were performed. The results indicated that these assays can be divided into groups. Thus, all tests belonging to different groups detect different antigenic molecules, each characterized by a specific combination of epitopes. Therefore, one assay was selected from each group for further experiments.

Although the tests are sensitive enough to allow quantification of antigens in unconcentrated urine, no significant amounts of antigens were detected in human serum. To find out whether urinary excretion is correlated with renal tissue damage, the normal range (mean ± 2.5 SD) of urinary antigen was determined by assaying the urine of healthy persons.

Antigen excretion in the urine of patients suffering from various kidney diseases was compared with the antigen excretion in the urine of healthy persons. Within the patients' group part of the individual values (10 to 30%) were significantly increased.

High levels of urinary antigens were observed in all cases of clinically diagnosed acute phases of renal diseases (acute pyelonephritis, urosepsis). Destruction of renal parenchyma in cases of hydronephrosis, nephrolithiasis, interstitial nephritis and polycystic disease of the kidney was always accompanied by increased release of antigens in urine. In addition, some of the patients suffering from chronic glomerulonephritis or diabetic nephropathy showed high urinary levels of antigens as well, which is indicative of tissue destruction.

These experiments have been described in detail recently (69–72). Some of the results obtained are shown in figures 2 and 3.

In figure 2 the excretion of 7 urinary antigens (UA 1–7), each defined by a certain combination of monoclonal antibodies, is measured in urinary samples of patients suffering from nephrolithiasis (fig. 2a) or pyelonephritis (fig. 2b).

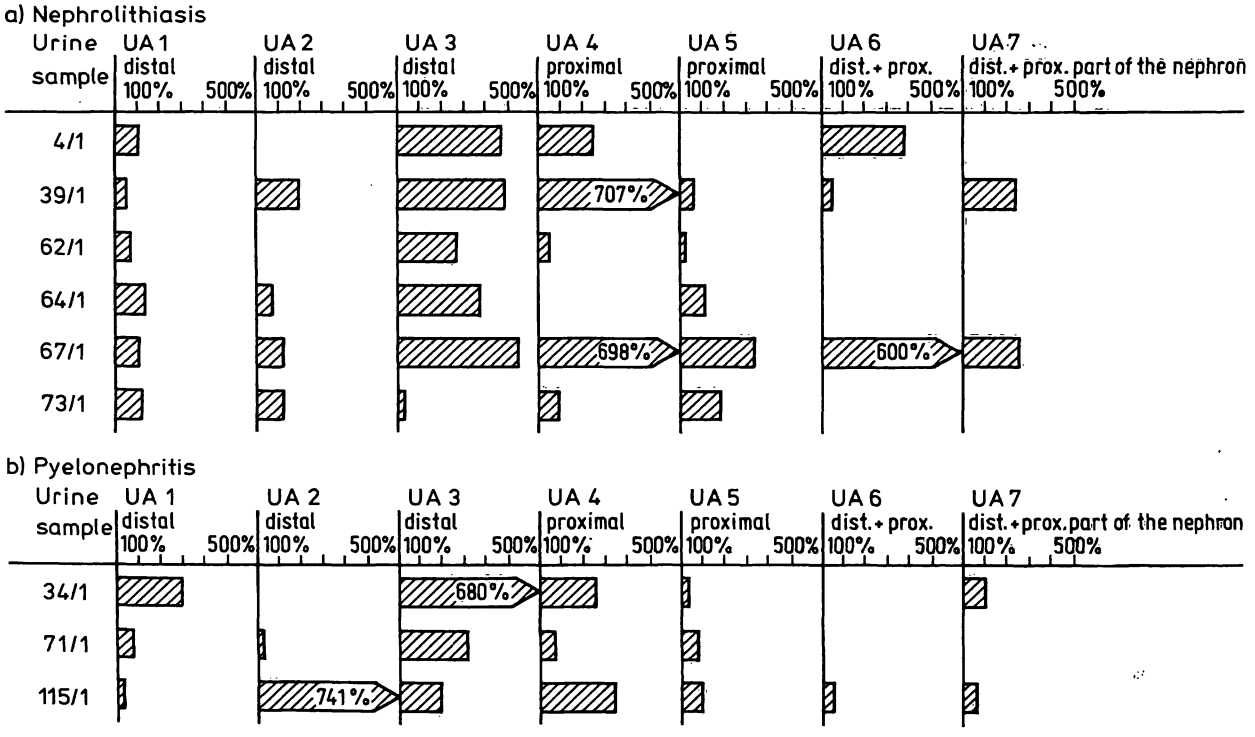

Fig. 2. Excretion of urinary antigens (UA) derived from distal (UA1, UA2, UA3) and proximal (UA4, UA5) part of the nephron or localized in all parts of the tubular system (UA6, UA7) in patients suffering from nephrolithiasis (fig. 2a) and pyelonephritis (fig. 2b). Antigen excretion is given as percent of upper limit of normal range (100% = mean + 2.5 × SD).

As expected, in such cases, in which the disease afflicts the distal part of the nephron primarily, excretion of distal tubular antigens is strongly enhanced. However, the proximal part of the nephron must be involved too, since antigens from this region also occur in the urine.

Having tested, in the meantime, urinary samples from several hundred patients with diseases related to the kidney, using 10 ELISA test systems based on 10 different combinations of monoclonal antibodies, the heterogeneity of the excretion pattern observed is confusing. Much work has still to be done to correlate antigen excretion with the clinically determined state of the disease.

In a follow-up study of a patient with only one kidney suffering from hydronephrosis (ureterolith), the excretion of a distal tubular and a proximal tubular antigen was monitored before and after removal of the stone. As can be clearly seen (figs. 3a and 3b), removal of the stone is immediately followed by the release of distal and proximal tubular antigens. After three days, antigen excretion returns to normal values. This antigen release is most probably caused by the washout of antigens from cells that have been damaged by the pressure built up by the obstacle in the ureter.

In figure 4 the immunohistochemical localization of two of the antigenic epitopes in the kidney is shown. The epitope recognized by monoclonal antibody PM II 80 F5 which is part of the UA4 antigenic particle is clearly localized in the luminal part of cells of the proximal tubule (fig. 4a). In contrast, the antigenic particle UA2 which carries an epitope recognized by the monoclonal antibody PM II 29 D2 is confined to distal tubular cells (fig. 4b). This latter antigen is distributed evenly in the tubular cells. There is no or only very weak overlapping of the staining patterns exhibited by the two monoclonal antibodies which were directly labelled with fluorescein (PM II 80 F5) and rhodamine (PM II 29 D2) and applied to the same tissue slice.

So far, the most important outcome of our studies is that it has become possible to monitor the excretion of antigens with clearly defined localization in the nephron. Correlation between antigen excretion and localization of the primary event in the tissue therefore becomes possible. Whereas similar test have been worked out for the proximal tubular part of the nephron, e.g. measurement of alanine aminopeptidase excretion (65), nothing is known about distal tubular markers. Through our tests it has become possible now to determine the excretion of *distal* tubular markers as well. In an attempt to identify

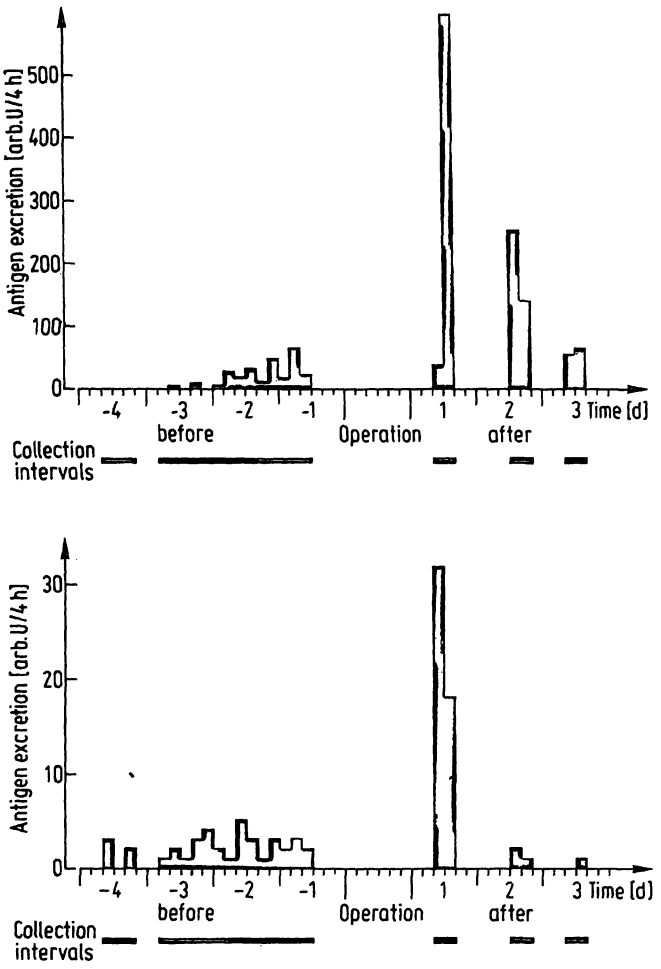

Fig. 3. Excretion of kidney-derived urinary antigens (UA) in the urine from a case of hydronephrosis (urethrolith).
a) Proximal tubular antigen UA4 defined by monoclonal antibodies PM II 35 H1 and PM II 80 F5 GOD.
b) Distal tubular antigen UA2 defined by monoclonal antibodies PM II 29 D2 and PM II 9 C2 GOD.
Antigen excretion is given as arbitrary units per collection interval (4 hours).

cellular breakdown products of tubular cells in the urine of patients treated with cis-platinum, we have used the immunogold method for ultrastructural identification. Distal tubular antigens carrying the PM II 9 C2 epitope were found in filament-like structures recovered from the urine by ultracentrifugation (72).

Monoclonal Antibodies of Other Species

Nearly all of the hybridoma work done up to date has made use of the well defined and almost foolproof mouse plasmacytoma cell lines as myeloma fusion partners. It was and still is difficult, however, to immortalize lymphoid cells of species other than mouse. Rat lymphocytes have been successfully fused to mouse myeloma cells. This technique of "in-

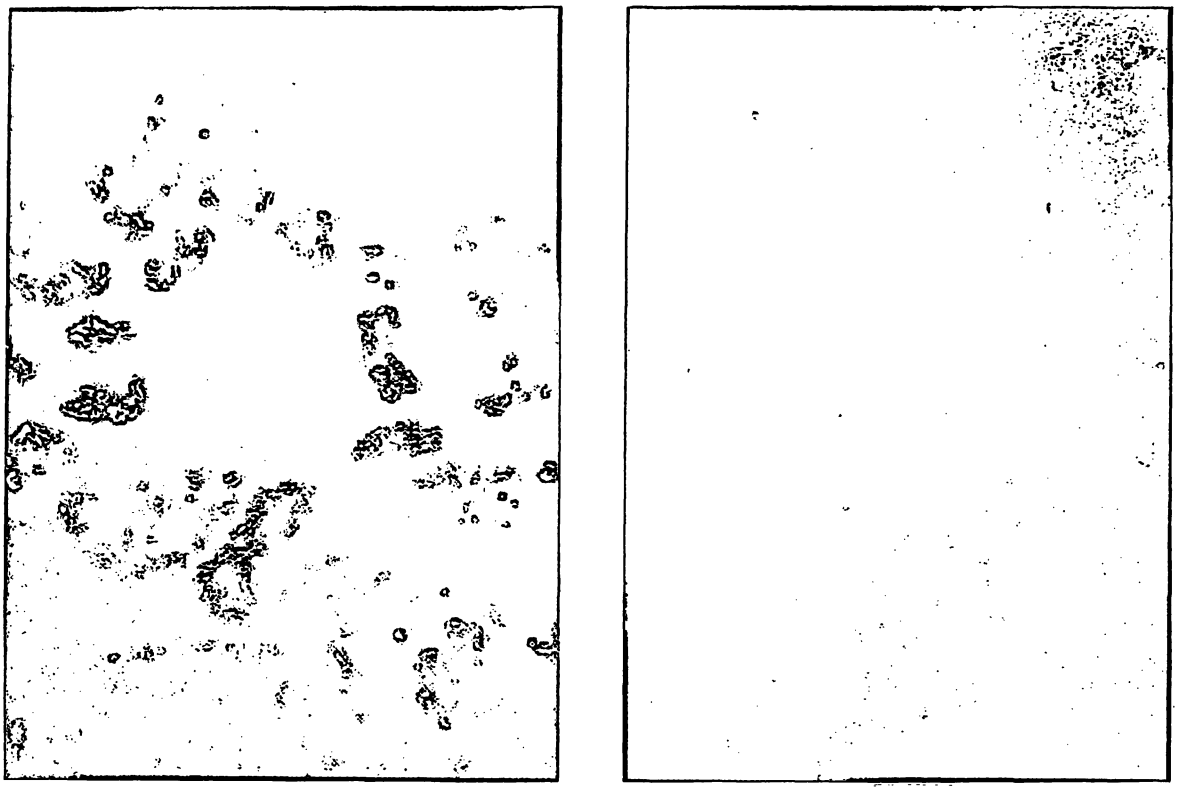

Fig. 4. Identification of localization of two antigenic epitopes in the same kidney slice by two directly labeled monoclonal antibodies.
a) Fluorescein labeled monoclonal antibody PM II 80 F5.
b) Rhodamine labeled monoclonal antibody PM II 29 D2.
Microphotographs were taken with an Olympus Fluorescence Microscope BH-2 using selection filters for each of the fluorochromes. Magnification 160×.

terspecies hybridization" works in principle with lymphocytes of all species. Whereas rat × mouse hybridomas are rather stable (73), products of fusion of mouse myeloma cells with lymphocytes from phylogenetically distant species such as man are rather labile and tend to lose mouse chromosomes and consequently their immortality.

Rat Monoclonal Antibodies

Two rat myeloma lines are now available to the scientific community, *Milstein*'s Y3-Agl.2.3 (74) and *Bazin*'s IR983F (75, 76). One of the advantages of using rat in addition to mouse myeloma lines for cell fusion is the expansion of the available repertoire of monoclonal antibodies, since the rat's immune system might recognize determinants completely different from those recognized by the mouse's immune system. Even more important is the observation that monoclonal rat antibodies easily activate human complement and, therefore, might have an advantage over monoclonal mouse antibodies for therapeutic purposes (77, 78).

Human Monoclonal Antibodies

The fusion of human lymphocytes with mouse plasmacytoma cells has in some cases been successful (79). *Schlom* et al. (80) reported the fusion of lymphocytes obtained from lymph node biopsy specimens from patients undergoing mastectomy for breast cancer. These lymphocytes were fused with mouse myeloma cells and yielded a few stable man × mouse hybridomas secreting antibodies specific for human breast cancer tissue components. These results suggest that the tumour elicits an autologous immune response in the cancer bearing patient.

The availability of stimulated lymphocytes of the B cell lineage from human donors is one of the preconditions for fusion of human cells. Peripheral lymphocytes are the most common source of such fusionable cells. They can be obtained from normal donors as well as from donors immunized by natural infection or by vaccination. Human monoclonal antibodies resulting from such fusion experiments would yield information on the monoclonal components of the human immune response to infectious agents. In addition, they might have advantages over polyclonal human antisera for therapeutic purposes.

The spectrum of human monoclonal antibodies obtainable by fusion of cells from immunized donors is, however, limited since humans cannot be immunized like laboratory animals. On the other hand, in vitro immunization of human lymphocytes still is a very complicated procedure and has only been successful with relatively few antigens (81). The peripheral blood of patients suffering from autoimmune diseases should contain fusionable lymphocytes. The immortalization of the monoclonal components of the humoral autoimmune response should yield valuable information on the antigens involved and on the pathomechanism of the disease. Anti-idiotypic antisera to such monoclonal autoimmune antibodies might one day help to regulate autoimmune diseases and to clear the blood of these autoantibodies through extracorporal immunosorption.

For the preparaton of human monoclonal antibodies to other antigens of interest, e.g. in cancer research, one is dependent on the patient's autologous immune response. If a patient has developed antibodies to his own tumour the corresponding fusionable B lymphocytes are available not only from the blood but also from draining lymph nodes or from the excised tumour itself (intra-tumoural lymphocytes).

There is a considerable body of evidence for the presence of autologous immune response to various types of malignant diseases. These data indicate that most tumour patients develop an autologous humoral immune response to their tumour. The question whether or not such immune responses have any impact on the tumour, either enhancing or inhibiting tumour growth, must at the moment remain unanswered. Irrespective of the role of the patient's immune response in the immunobiology of the tumour, the future potential of immortalization and dissection of such autologous immune responses is a challenge as well as a promise for immunologists.

For the successful fusion of human lymphocytes, a human myeloma line, comparable in its properties to the mouse myeloma lines, is urgently needed. So far most of the reports on successful fusions with human myeloma lines have to be taken with great reservation and doubt (82, 83). The usefulness of the available human plasmacytoma or lymphoblastoid cells line for fusion purposes was reviewed by *Kozbor* et al. (84) and by *Abrams* et al. (85). In all cases, mycoplasmal infections might cause the most severe problems in human hybridoma technology. Therefore, availability of mycoplasma free fusion partners (both lymphocytes and myeloma cells) seems to be the most important precondition for successful human × human fusions (86).

In principle the results obtained so far seem promising and should encourage other investigators to develop this important field of hybridoma technology. A patient's immune response against his own tumour should be more specific than the immune response of an immunized animal can be expected to be. If not for therapeutic, then definitely for diagnostic purposes, human monoclonal antibodies will have a great future potential.

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Radioimmunoassay: An Overview[1])

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Summary: The historical development, principle and theory of the radioimmunological test are described, together with the necessary test components, separation techniques and experimental procedure. The emphasis on different areas of diagnosis that rely on radioimmunoassay is compared for two institutions.

Methods for the determination and presentation of reliability criteria are described, and the current status of quality control of radioimmunological methods is discussed.

Three high grade mechanized systems and five fully mechanized or automated systems for radioimmunological assays are presented.

Future trends in the development of radioimmunoassay can be predicted with the aid of general growth criteria.

Radioimmunoassay: Ein Überblick

Zusammenfassung: Historische Entwicklung, Prinzip und Theorie des radioimmunologischen Tests werden beschrieben, Anforderungen an Testkomponenten, Trenntechniken und Durchführung werden dargestellt. Aus dem Anwendungsbereich des RIA werden klinisch-diagnostisch wichtige Schwerpunkte anteilig für zwei Institutionen gegenübergestellt. Methoden zur Ermittlung und Darstellung von Zuverlässigkeitskriterien sowie der aktuelle Status der Qualitätskontrolle radioimmunologischer Verfahren werden beschrieben.

Drei hochgradig mechanisierte Systeme zur Durchführung radioimmunologischer Tests sowie fünf vollmechanisierte bzw. automatisierte Systeme werden dargestellt.

Entwicklungstendenzen des Radioimmunoassays, die anhand allgemeiner Wachstumskriterien erkennbar sind, werden gegeben.

History

In 1952, *Arthur Mirsky* proposed that maturity onset diabetes could be attributed not so much to a defect of secretion, but rather to an accelerated degradation of insulin by "insulinase" of the liver (1). In order to test this hypothesis, *Yalow & Berson* investigated the metabolism of ^{131}I-labelled insulin, and observed that it was eliminated more slowly by insulin-sensitive diabetics than by non-diabetics (2). They explained this delay by the binding of labelled insulin to an insulin-binding globulin fraction. Using isotopic tracing and radiochromato-electrophoresis, they showed that this globulin fraction occurred generally in the plasma of insulin-sensitive diabetics, and suggested that it contained an acquired insulin antibody. To the immunologists in the mid fifties, however, this was an unacceptable concept, and the fundamental studies of *Yalow & Berson* were reject-

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

ed by "Science" and "Journal of Clinical Investigation" in 1955 (3). The work was accepted in 1956, but only after a compromise has been reached with the editors, in which the term "insulin antibody" was removed from the title. In the same publication they also reported that the quantity of labelled insulin bound to a defined concentration of antibody was related to the actual insulin concentration. This observation formed the basis for the radioimmunological detection of plasma insulin, and for the subsequent development of all radioimmunological and related methods of saturation and displacement analysis. Adoption of the method was, at first, extraordinarily slow. Bioassays were still the preferred methods for hormone assays. No great interest was shown in the radioimmunological test until four years later, when *Yalow & Berson* described the measurement of plasma insulin without extraction (4). Slowly, it became recognized that radioimmunoassay was a highly sensitive and comparatively simple method for performing assays in small samples and in large sample series.

Even up to the mid sixties, *Yalow & Berson* were practically the only authors using this method. Five years later, however, in 1970, radioimmunoassay had already developed into the most important and comprehensive analytical method in the endocrinological laboratory. It then spread rapidly into medicine and veterinary medicine, and into nonmedical areas concerned with the analysis of low concentrations of organic compounds (fig. 1). In 1974, radioimmunological methods were performed in about 2000 laboratories in the USA, increasing in 1975 to more than 4000 laboratories. Meanwhile, the fundamental work of *Yalow & Berson* in 1960 (4) has become a classic reference. The increase in the number of radioimmunological determinations since 1975 is shown in figure 2 (5—8).

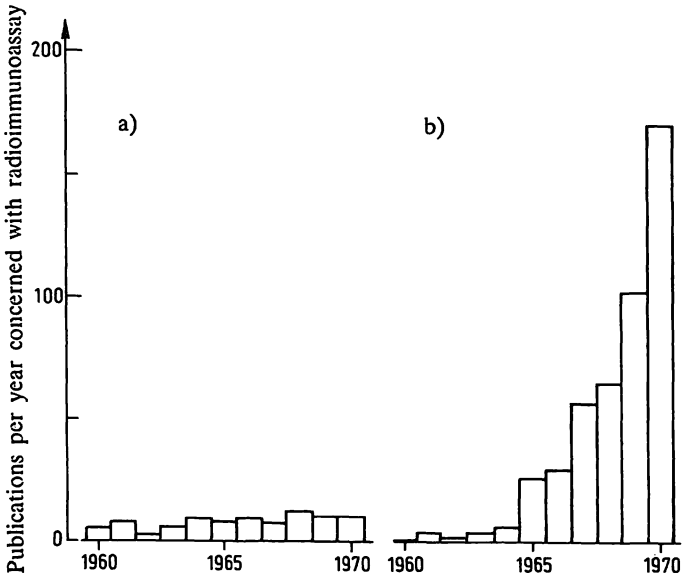


Fig. 1. Increase in the number of publications concerned with radioimmunoassays.
a) Yalow, R. S. & Berson, S. A. (1960) J. Clin. Invest. *39*, 1157. Number of times quoted: 1100 (1961—1975).
b) Journal of Clinical Investigation,
Diabetes,
Journal of Clinical Endocrinology,
Endocrinology

	RIA-Tests	
	USA	World-wide
1975	–	52000000
1976	52000000	100000000
1980	100000000	200000000
1985	>200000000	>400000000
	Federal Republic of Germany	
1979	17000000	
1985	39000000	

Fig. 2. Growth in the number of radioimmunological tests.

Principle and Theory

The principle and theory of the radioimmunological test are comparatively simple and straight forward, and therefore offer no basis for understanding the less than satisfactory precision and accuracy of the method that still exist today. In the radioimmunological system (fig. 3), antigen (AG) and antibody (AB) interact reversibly to form a soluble antigen-antibody complex. Labelled antigen (AG*) and unlabelled antigen compete for binding to the limited number of antibody binding sites. The process obeys the law of mass action, so that the greater the quantity of unlabelled antigen present, the less labelled antigen is bound. Since labelled antigen and unlabelled antigen are in excess of the antibody (i.e. [AG*] and [AG] in excess of [AB], where [] refers to the concentration of valencies, rather than the molar concentration), the term, saturation analysis, is also used. The concentration of unlabelled antigen is derived from the extent to which it competitively inhibits the binding of radioactive antigen to a specific antibody, the method being standardized with known concentrations of unlabelled antigen. To perform this typical comparative in vitro analysis, known concentrations of antibody and labelled antigen are incubated at a defined temperature with various concentrations of unlabelled antigen (standard

or sample), usually until equilibrium is reached. Free antigen is then separated from bound antigen, and the radioactivity of one or both fractions is measured. If an homogeneous antibody with equal affinity for labelled and unlabelled antigen were used, and the reaction allowed to attain saturation, then the standard curve would be an hyperbola (B/B_0 = f([AG] + [AG*])), which could be transformed to a linear, double reciprocal or double logarithmic plot (figs. 3a and 3b). In some cases, the standard curve obeys a mathematical model, which suffices for the binding kinetics and the detection limit (*Scatchard* plot, quadratic equation, hyperbola, logit-log transformation, etc.). This is not usually the case, however, so that purely empirical interpolation methods are used for curve fitting (parabola, polynomials of various degrees, smoothed spline approximation, etc.) (9, 10).

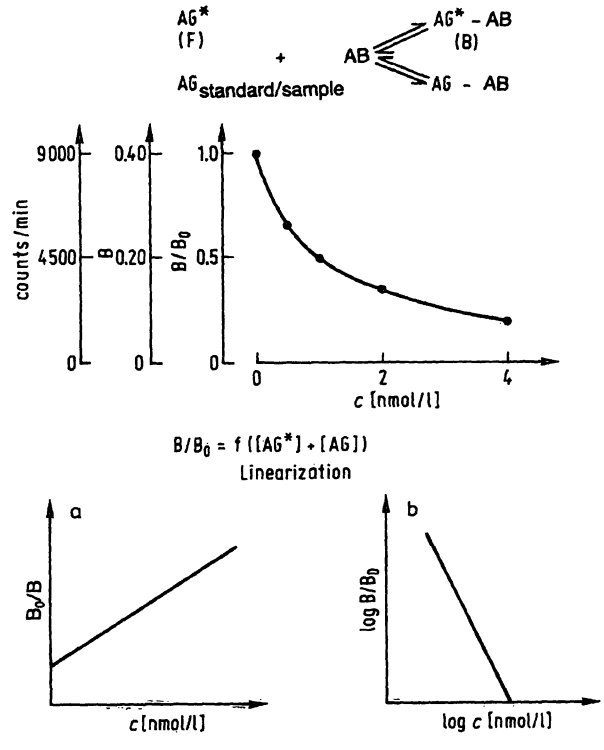


Fig. 3. Principle of the radioimmunological test

Test Components

Antigens and antibodies

An antigen is any substance that binds to an antibody. Large antigens are immunogens per se. If the molecule is too small or lacks sufficient chemical differentiation to act as an antigen, it is coupled covalently to a higher molecular weight carrier (serum albumin possesses a large number of different reactive groups that can be exploited for this purpose), and the conjugate is used for immunization. To ensure that the assay is free from interference, it is essential that the standard, the antigen used for the immunization and the labelled antigen be very pure.

The properties of an antiserum raised against an hapten-carrier conjugate are predetermined by the type of carrier and by the site of coupling between hapten and carrier, whereas the properties of an antiserum raised against a high molecular weight antigen are rather unpredictable. Especially in the measurement of pharmaceuticals and their metabolites, it must be decided whether to use an antiserum that recognizes a whole family of molecules (original compound and its metabolites) or one that recognizes only biologically active metabolites.

Qualitative and quantitative evaluation of an antibody for use in a radioimmunoassay is based on the two criteria of specificity and affinity. Specificity is determined from cross reaction with antigen analogues (see also the section on Reliability), and affinity is determined by measurement of affinity constants. The stability of antigen-antibody complexes is larger for multivalent antigens, owing to the augmented formation of an intermolecular network. In such cases the antigen-antibody reaction is more appropriately characterized by the overall, average affinity constant, known as the avidity constant. This effect of antigen valency seems to be the main reason for the greater sensitivity of immunological tests for proteins and polypeptides, compared with those for haptens.

To a first approximation, the sensitivity of a test, i.e. the minimal detectable concentration, corresponds to $1/K_a$ or $1/K_{av}$, where the affinity constant, K_a, for the hapten-antibody interaction is normally in the range 10^6–10^{10} l/mol, and K_{av} for protein-antibody interaction can be as high as 10^{13} l/mol (fig. 4).

$$[AG] + [AB] \underset{k'}{\overset{k}{\rightleftharpoons}} [AG - AB]$$

Free (F) Bound (B)

$$K_a = \frac{k}{k'} \qquad [AB_0] = [AB] + [AG - AB]$$

$$B/F = K_a([AB_0] - B)$$

1. $[AB_0] \gtrsim B$: $\frac{\Delta B/F}{\Delta B} = \max$ ($\triangleq$ high sensitivity)

2. $B \lesssim [H_{min}]$

from 1. and 2.: $[AB_0] \simeq [H_{min}]$

when $B/F = 1$: $1 \lesssim K_a[AB_0] \lesssim K_a[H_{min}]$

$$[H_{min}] \gtrsim \frac{1}{K_a}$$

Fig. 4. Detection limit – affinity constant.

Labelled antigen (tracer)

The measured parameter in the radioimmunoassay is the radiation of a nuclide, which must be introduced into the antigen molecule. Suitable nuclides may be isotopes of normal component atoms of the antigen, which can be introduced by exchange or attached to the antigen in the form of an immunologically inert residue; or they may be foreign atoms with especially favourable isotopic properties, and likewise introduced by exchange or coupled to the antigen with an extra residue.

To guarantee a sufficiently sensitive test for use in a defined area of biological enquiry, the isotope must have a certain minimal specific activity. This is illustrated by a survey of the factors that govern the required specific radioactivity of insulin for use as a marker antigen (fig. 5). Taking into consideration the chemical feasibility of introducing the nuclide into a variety of antigen structures, as well as the half life of the nuclide, only three isotopes are obviously suitable candidates for antigen labelling; these are the iodine isotopes, ^{131}I and ^{125}I, and tritium, ^{3}H, although the use of ^{131}I is limited on account of its short half life (fig. 6).

Plasma insulin concentration
10 mU/l ≙ 0.4 µg/l ≙ 70 pmol/l

Detection of: 7 pmol/l
for high affinity AB: [AG*]:[AG] ≃ 10:1 ($\Delta B/B_0 \simeq 5\%$)

[AG*] ≃ 70 pmol/l
70 pmol ≙ $3 \cdot 10^6$ disintegrations per min ($1-2 \cdot 10^6$ counts/min)

Specific activity: 714 GBq/mmol (124 MBq/mg)
(19.3 Ci/mmol (3.35 mCi/mg))

Isotope:Insulin = 1:1
from: maximal specific activity =

$$\frac{3.56 \cdot 10^5}{t_{1/2\,(\text{years})}}\ [\text{Ci/mol}]\ (\times 37 = \text{GBq/mol})$$

$t_{1/2}$ of isotope $\lesssim$ 18.5 years

Fig. 5. Relationship between detection limit and the nuclide half life.

Tritium labelling is performed by metal-catalysed hydrogenation of unsaturated compounds, by the metal-catalysed exchange of halogens or hydrogen for tritium, or by the reduction of oxidized intermediates with tritiated metal hydrides. Iodine labelling with $Na^{125}I$ involves direct iodination of tyrosine and sometimes also histidine residues, using the iodine monochloride method, the chloramine T method, the electrolytic method or the lactoperoxidase method. In indirect iodination, the antigen is covalently bound to an iodinated accessory molecule, e.g. histamine, tyrosine methyl ester, or tyramine; or it is reacted with the iodinated N-hydroxy-succinimide ester of *p*-hydroxy-phenylpropionic acid, i.e. the *Bolton-Hunter* reagent (11).

Isotope	Half life	Maximal specific activity (Ci/m Atom)	(Bq/m Atom)
^{131}I	8 days	16000	592×10^{12}
^{32}P	14.3 days	9200	340×10^{12}
^{125}I	60 days	2200	81×10^{12}
^{35}S	87 days	1500	56×10^{12}
^{3}H	12.3 years	30	1110×10^{9}
^{14}C	5730 years	0.062	2294×10^{6}

Fig. 6. Nuclides used in in vitro diagnostic methods.

Separation Methods

An essential step in the radioimmunological assay is the separation of the free and the antibody-bound, labelled antigen. With the exception of the Internal Sample Attenuator Counting method (12), all present day radioimmunological assays are heterogeneous methods. The two fractions are therefore finally separated by physical methods (centrifugation, filtration, decantation, electrophoresis, chromatography) (fig. 7). For an ideal separation, the following conditions must be satisfied:

1. quantitative separation with no effect on the antigen-antibody reaction (guarantee of high precision and sensitivity),
2. simple, rapid and cheap performance (high practicability),
3. no influence by plasma components (high reproducibility and comparability) (13, 14),
4. no unspecific effects (15, 16), and
5. time-dependent stability of the antigen-antibody complex under the separation conditions (mechanized, sequential systems) (17, 18).

Principle	Method/Material
Mobility	Chromatoelectrophoresis, electrophoresis, gel filtration, chromatography
Adsorption	Dextran (gelatin)/active charcoal, silicates, ion exchangers, cellulose
Fractional precipitation	Organic solvents, polyhydric alcohols, salts, acids, double antibodies (poly-ethyleneglycol), Staphylococcal protein A
Solid phase	Antibody coating: reaction vessels, capillaries, agarose, cellulose, Sephadex, Sepharose, polymer-encapsulated iron Double antibody coating: Sepharose, cellulose, etc.
Internal sample	Agarose/bismuth oxide/active charcoal particle
Attenuator counting	Agarose/bismuth oxide/antibody particle Cd, W, Pb, Bi, Hg? Particle size sedimentation? % Absorption of radiation?

Fig. 7. Separation techniques in radioimmunoassay.

Non-Equilibrium Radioimmunoassay

Basically, it is possible to perform the test before binding has reached equilibrium. Since the rate of association at constant antibody concentration depends on the antigen concentration, equilibrium is reached in the flat, terminal part of the standard curve sooner than in the initial part. If standards and samples are treated identically, the test can be conducted according to this principle, although the detection limit is decreased as shown by the flattening of the initial part of the standard curve (fig. 8).

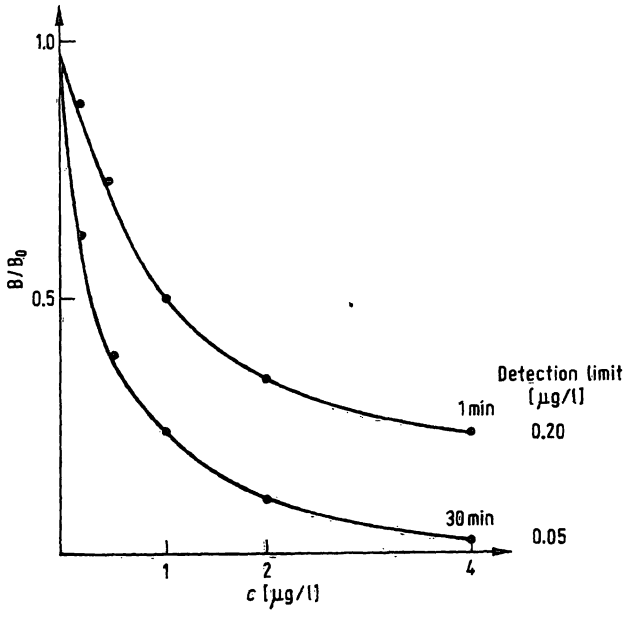


Fig. 8. Non-equilibrium radioimmunoassay (digoxin).

If the equilibrium constant of the reverse reaction (dissociation of the antigen-antibody complex) is small relative to the association constant, then the detection limit can be increased by first exposing the antibody to unlabelled antigen until optimal binding is attained, followed by incubation with the labelled antigen.

Application

In principle, radioimmunoassay can be used for the quantitative determination of any substance that is available in pure form, and to which an antibody can be raised. More than 400 substances of widely varying chemical types and biological importance have now been measured by this technique, chiefly in the fields of medical diagnosis and clinical chemical research. About 100 of these are essentially important in routine diagnosis; following a suggestion of *Rosalyn Yalow*, they can be divided into three classes: peptide hormones, non-peptide hormones and non-hormonal substances (3).

The majority of the substances shown in figure 9 can be assigned to five crucial areas of diagnosis: thyroid, hepatitis, cancer, *Digitalis* glycosides and general diagnosis. In figure 10, the percentage contribution of each of these five areas is shown for Met Path, one of the largest clinical chemical laboratories in the USA (calculated from figures for September 1977), and for the Medical School in Hannover (average figures for 1982). The apportionment shown here for the Hannover medical school is possibly not strongly representative of other clinics in the Federal Republic of Germany, because considerable shifts of emphasis may be caused by local factors (a large women's hospital and a special section for diabetes cause a disproportional increase in sector G), and by state political or legislative measures (neonatal hypothyreosis screening is not included in sector T).

Reliability

It is the purpose of all clinical chemical investigations, including those performed by radioimmunoassay, to provide reliable analytical results as a basis for reliable clinical diagnosis. Reliability depends on the type and degree of incidental and systematic errors, which should be monitored, identified and classified by an internal laboratory quality control system, the long term aim being to decrease or abolish such errors. Since radioimmunoassay is a multistep procedure, the possibilities for error are correspondingly large (fig. 11).

Peptide hormones	Non-peptide hormones	Non-hormones
Hypophysis Human somatotropin, corticotropin, α-melanocyte stimulating hormone, β-melanocyte stimulating hormone, thyrotropin, follitropin, lutropin, prolactin, β-lipotropin, antidiuretic hormone, ocytocin	*Thyroid* 3,5,3′-triiodothyronine, thyroxin, reverse triiodothyronine	*Pharmaceuticals, vitamins* Cardiac glycosides, drugs, psychoactive agents, antibiotics, tricyclic antidepressants, vitamin B_{12}, folic acid
Placenta Human chorionic gonadotropin, human chorionic somatomammotropin	*Steroids* Aldosterone, corticosteroids, oestrogens, androgens, progesterone	*Cyclic nucleotides, enzymes* C1-Esterase, fructose-1,6-bisphosphatase, plasminogen, plasmin, chymotrypsin, trypsin, carbonic anhydrase, aldose reductase, carboxypeptidase B, pancreatic elastase, creatine kinase isoenzymes, acid phosphatase
Pancreas Insulin, glucagon, C-peptide, proinsulin, pancreatic polypeptide	*Prostaglandins* *Leukotrienes*	*Viruses, viral proteins, bacterial antigens, parasite antigens, tumour antigens* Carcino-embryonic antigen, α-foetoprotein, alkaline phosphatase, pro-adrenocorticotropic hormone, ferritin, coeruloplasmin, tissue polypeptide antigen, β-microglobulin, foetal sulphoglycoprotein, lung tumour associated antigen.
Calcitropic hormones Parathyrin, calcitonin	*Biogenic amines* Serotonin, melatonin, catecholamines	*Serum proteins* Thyroxin-binding globulin, albumin, IgG, IgE, IgA, IgM, properdin, fibrinogen, apolipoproteins, myoglobin
Gastrointestinal tract Gastrin, secretin, cholecystokinin, vasoactive intestinal peptide, gastric inhibitory peptide, motilin, neurotensin, enteroglucagon		*Other substances* Rheumatoid factors, coagulation factors, complement factors, neurophysins, endotoxins
Vasoactive tissue hormones Angiotensins, bradykinins		
Liberins, statins Thyroliberin, luliberin, somatostatin		
Other peptides Substance P, endorphins, enkephalins, thyroglobulin		

Fig. 9. List of some substances that can be determined by radioimmunoassay.

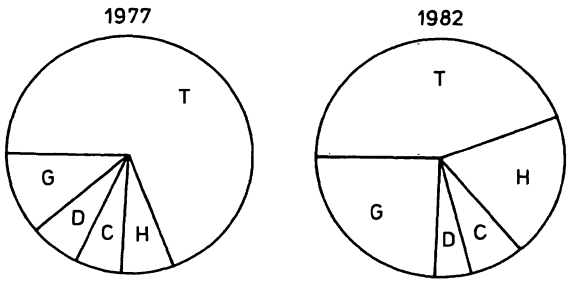


Fig. 10. Proportions of radioimmunological determinations in the sectors: thyroid (T), hepatitis (H), cancer (C), *Digitalis* glycosides (D) and general diagnosis (G)
1977 = Met Path, USA
1982 = Medizinische Hochschule Hannover

Reliability can be determined with the aid of the defined and quantifiable reliability criteria: detection limit, specificity, precision and accuracy. Detection limit or sensitivity is limited essentially by the affinity constant of the antibody, and can be determined in various ways (fig. 12):

1. According to *Ekins* (20) it is determined from the initial slope of the standard curve, thus representing a special case of *Ekins'* precision concept: Δc (= precision of the measurement of c) depends on the slope and the error of the measurement of B, i.e. ΔB; if $c = 0$, the corresponding value of Δc, i.e. Δc_0, defines the detection limit.
2. *Schwarz*'s method for internal quality control. This is based on a logit-log transformation, and (because of heteroscedasticity i.e. irregular distribution of variance over the standard curve) it uses only that part of the standard curve between 15 and 85% of the initial binding of unlabelled antigen (B_0). As an operational definition, test sensitivity is therefore $0.85 \times B_0$ (21).
3. As a general guide for ensuring the quality of clinical chemical results, the dection limit can be taken as $B_0 - 3s_{B_0}$.

Component or step of a radioimmunological method	Variable factors that can cause differences in results
1. Reagents	
Standard antigen	Structure, purity, stability, protein binding
Labelled antigen	Type of labelling, specific radioactivity, stability, physical-chemical properties compared with those of the standard antigen
Antibody	Type of immunogen, species, immunization protocol, affinity constants for labelled and unlabelled antigen
Buffer system	Composition, ionic strength, pH, type and concentration of protective proteins, addition of other reagents (e.g. sodium azide, enzyme inhibitors)
Standard matrix	Composition
2. Sample matrix	Composition (unphysiological concentration of physiological components, different concentrations of unphysiological components)
3. Sample preparation	Isolation, storage, transport, pretreatment (extraction, heat treatment, anticoagulants, etc.)
4. Aliquotting and dilution	Quality of delivery system, i.e. mechanized or manual, materials.
5. Reaction vessel	Material, surface to volume ratio
6. Incubation	Temperature, time
7. Separation	Type of bound/free separation, effects of constituents of the sample matrix, unspecific binding
8. Radioactivity measurement	Type and quality of the counter, statistical counting errors
9. Data valuation	Type and quality of the fitting procedure, individual cases of unspecific binding

Fig. 11: Sources of error in radioimmunoassay.

Specificity is a measure of the exclusive nature of the binding between the antiserum and the antigen under investigation. The reciprocal term, cross reactivity, refers to antibody heterogeneity (= heterogeneous cross reactivity) or, as in the case of monoclonal antibodies, it refers to a limited ability of the antigen binding site to exclude cross reacting ligands (= homogeneous cross reactivity).

In conventional radioimmunoassay, heterogeneous and homogeneous cross reactivities cannot be differentiated, but differentiation is possible by using the studied antigen only in its labelled form. For two cross reacting antigens, two situations can be differentiated by a *Scatchard* plot (fig. 13):

1. saturable, high affinity binding of both labelled antigens (AG_1^* and AG_2^*) with equal affinities, in which the different binding capacity is indicative of a fraction of low capacity anti-AG_1^* within the anti-AG_2^* population (fig. 13a) (typical of an heterogeneous antiserum), and
2. a different affinity of AG_1^* and AG_2^* with equal binding capacities (typical of an homologous antiserum).

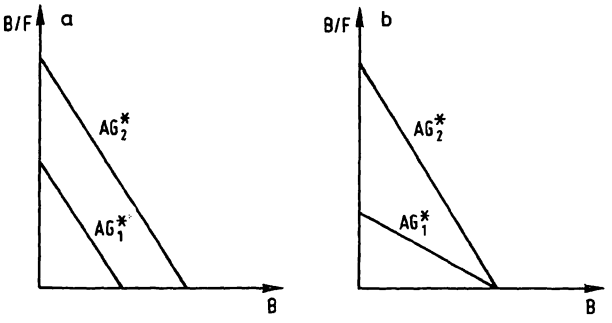


Fig. 13. *Scatchard* plots for the interaction of heterogeneous and homogeneous antisera with cross reacting antigens.
a) heterogeneous antiserum
b) homogeneous antiserum

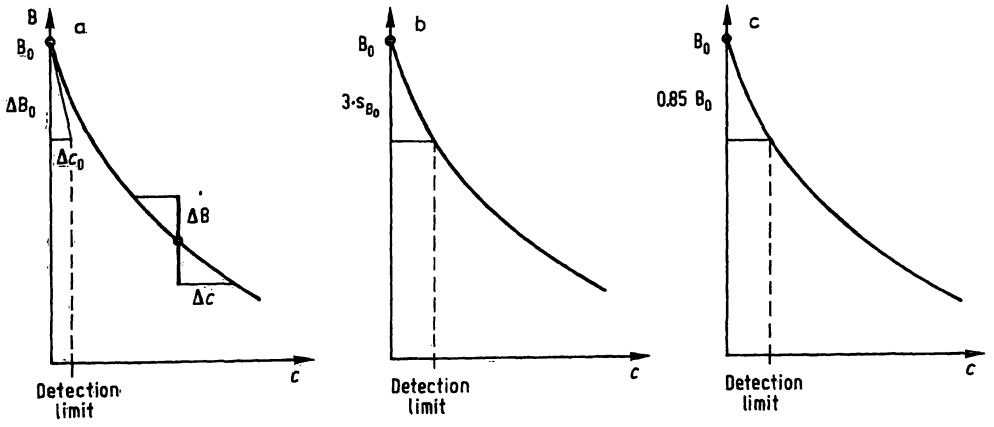


Fig. 12. Detection limit in radioimmunoassay.
a) after *R. P. Ekins*, ΔC = factor (slope, ΔB).
b) according to the guide lines for internal quality control of the German Society for Clinical Chemistry, $B_0 - 3 \cdot s_{B_0}$.
c) after *S. Schwarz*.

The common influences of incidental and systematic errors on the precision and accuracy of the radioimmunoassay suggest that they are related. This is clear from a comparison of two methods (fig. 14) (21): method A with a wide scatter (= incidental errors, poor precision, high imprecision factor) around the true value of ā, and method B with low scatter (good precision) about an average value, b̄, which differs from the true value (= systematic error). The quality of the analytical results from the two methods can be compared by using *Ekins*' equation (fig. 14). On the basis of its clinical value, method B would always be preferable (21).

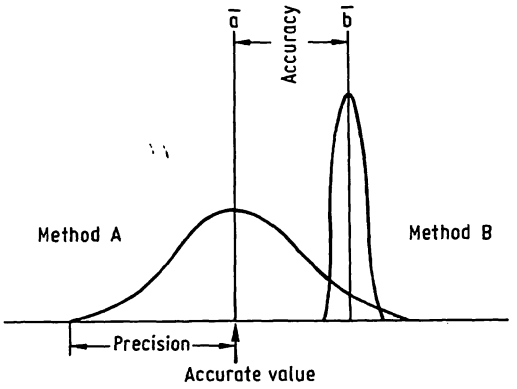


Fig. 14. Relationship between precision and accuracy.
Analytical quality = $\sqrt{\text{accuracy}^2 + \text{precision}^2}$ (after *Ekins*)

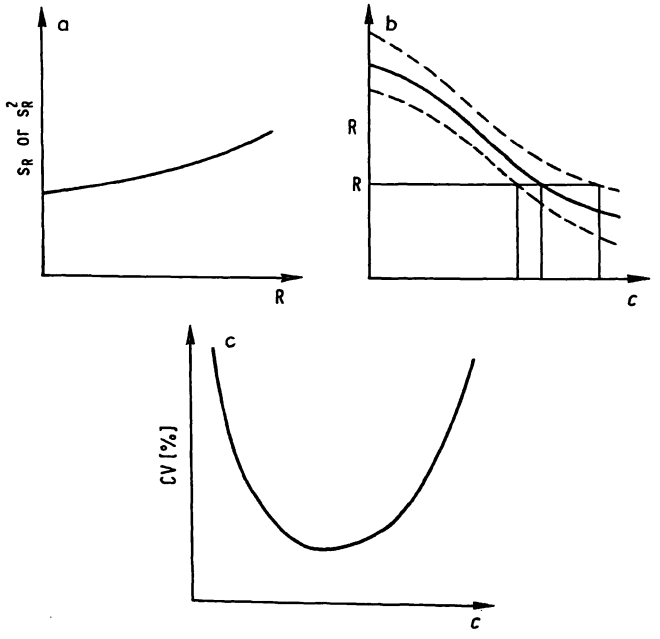
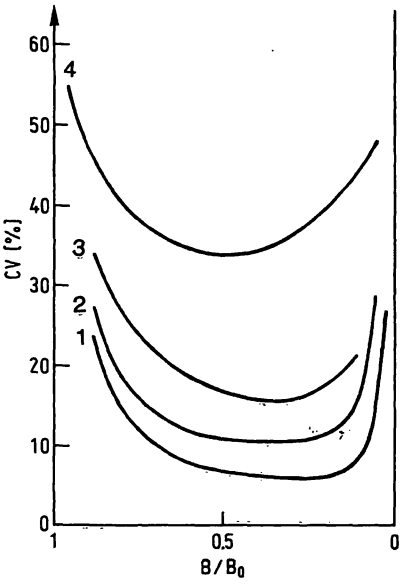


Fig. 15. Precision analysis of radioimmunological methods.
a) Response-error-relation
b) Standard curve
c) Precision profile
R = Replicate (mean)

In internal laboratory quality control, precision and accuracy are monitored separately. Nowdays, the normal procedure for precision analysis is to determine a response-error relationship (RER), which is represented by the standard deviation of replicates (usually duplicates), or their deviation from the average of the replicates. Taking into account the confidence range for antigen concentrations determined from the standard curve, a precision profile is then constructed. For a symmetrical distribution of the confidence limits, this profile then represents the dependence of the variation coefficient on the antigen concentration or the appropriate response parameter, and it shows how the precision alters over the range of the standard curve (fig. 15).

Precision profiles can be constructed for four different levels of precision (fig. 16) (21):



4. between laboratories

$$\% \text{ CV} = \frac{s_{mean}}{\overline{EQCS}} \cdot 100$$

3. inter-series

$$\% \text{ CV} = \frac{s_{mean}}{\overline{IQCS}} \cdot 100$$

2. within series

$$\% \text{ CV} = \frac{s_{Repl.}}{\bar{x}} \cdot 100$$

1. standard curve and fit

$$\% \text{ CV} = \frac{\sqrt{B^{1.5}}}{B/B_0 - (B/B_0)^2} \cdot \frac{\sqrt{MRV} \cdot \ln 10}{B_0 \cdot \text{slope}} \cdot 100$$

Fig. 16. Precision profile of the radioimmunoassay.

1. for errors that arise from the preparation of the standard curve and the curve fitting procedure,
2. for the level of precision within the series for replicate measurements,
3. for the level of precision between series for the measurement of an internal quality control sample, and
4. for the level of interlaboratory precision for the measurement of external quality control samples in interlaboratory collaborative investigations.

As a means of guaranteeing the internal quality of results, the type 3-level of precision is important. It comprises the total variance of the standard curve, the fit and the sample, and the interseries deviation. For radioimmunological methods it is found to be between 7 and 20% when the optimal range of the calibration curve is used for standardization.

Of the reliability criteria, accuracy contains the greatest element of uncertainty, as evidenced by the difference between the measured and true value of the antigen concentration. Definitive methods, which can be used to check the radioimmunological methods, are available for very few antigens (e.g. gas chromatography-mass spectrometry for steroids). For the majority of antigens, however, especially peptide and proteo-hormones, only the "relative internal" accuracy is monitored, using recovery experiments and/or the results are compared with those from already established (possibly performed in reference laboratories) radioimmunological methods.

In addition to work at the Institute for Clinical Biochemistry at the University of Bonn, contributions to improving the comparability of results between laboratories have been made in the last 10 years particularly by the group of *Marschner*, *Scriba* and *Wood*, using their own type of interlaboratory collaborative study. By identifying sources of error and by making educative recommendations to manufacturers and users of kits, they have helped to bring about a considerable increase in interlaboratory precision (fig. 17). Last year, *Marschner* presented the evidence for this improvement, using the example of thyroid stimulating hormone determination methods in the Federal Republic of Germany (22). Also last year, the World Health Organization presented its experience of the previous five years with the "matched reagents program", which involved more than 80 laboratories in 35 countries (23). Over the five year period, only one hormone assay (that for luteinizing hormone) out of the seven studied showed significantly better interlaboratory precision when performed with WHO reagents and protocols, than when performed with non-WHO reagents. If, however, one assesses the changes in interlaboratory precision over the five year period for both WHO and non-WHO methods, as shown in figure 18, there must be some doubt about the efficiency of the World Health Organization programme.

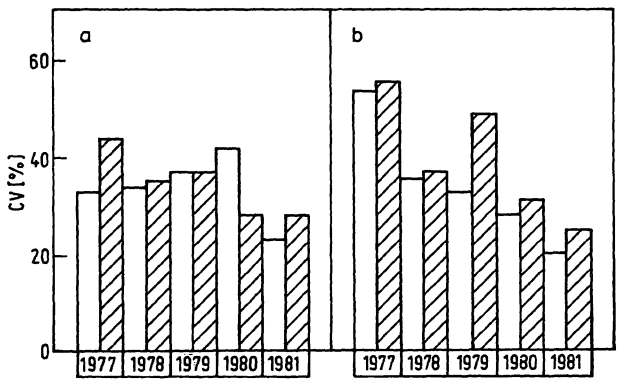


Fig. 18. Developments in precision for the determination of follitropin (a) and lutropin (b), using the 'matched reagents program' of the World Health Organization.
□ WHO reagents
▨ other

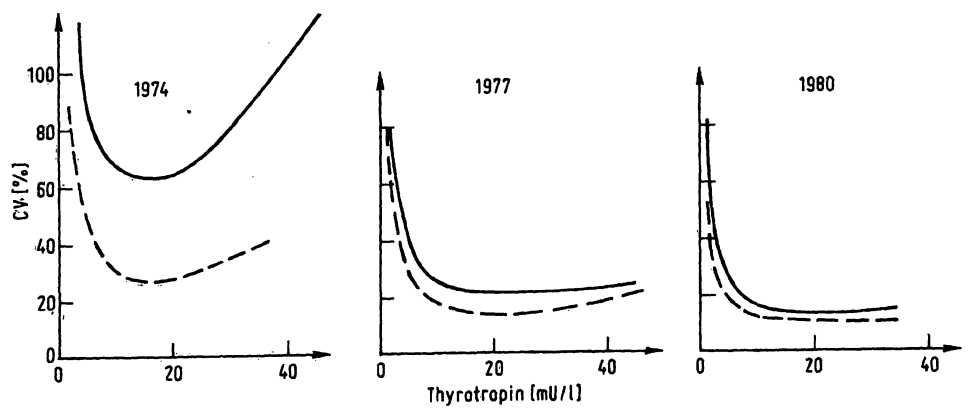


Fig. 17. Improvement in the precision of the assay for thyroid stimulating hormone in the Federal Republic of Germany.

A series of interlaboratory investigations have been conducted in England, the Federal Republic of Germany, the German Democratic Republic, Brazil and Italy since 1979 (24–29). The results have sometimes been poor (interlaboratory coefficients of variation of 20–70%), thus demonstrating how necessary it is to seek new concepts for the improvement of this aspect of radioimmunoassay. At present, the quality of every single radioimmunological method is such that it is used under regulations for exemption from the German standardization laws (30).

Mechanization and Automation

In routine analysis, there is a critical sample frequency above which the necessity for mechanization or automation is indicated. At the beginning of the seventies, this situation existed for certain radioimmunoassays.

Disregarding any special stages of sample preparation, the conventional manual radioimmunoassay consists of the following sequential steps (fig. 19):

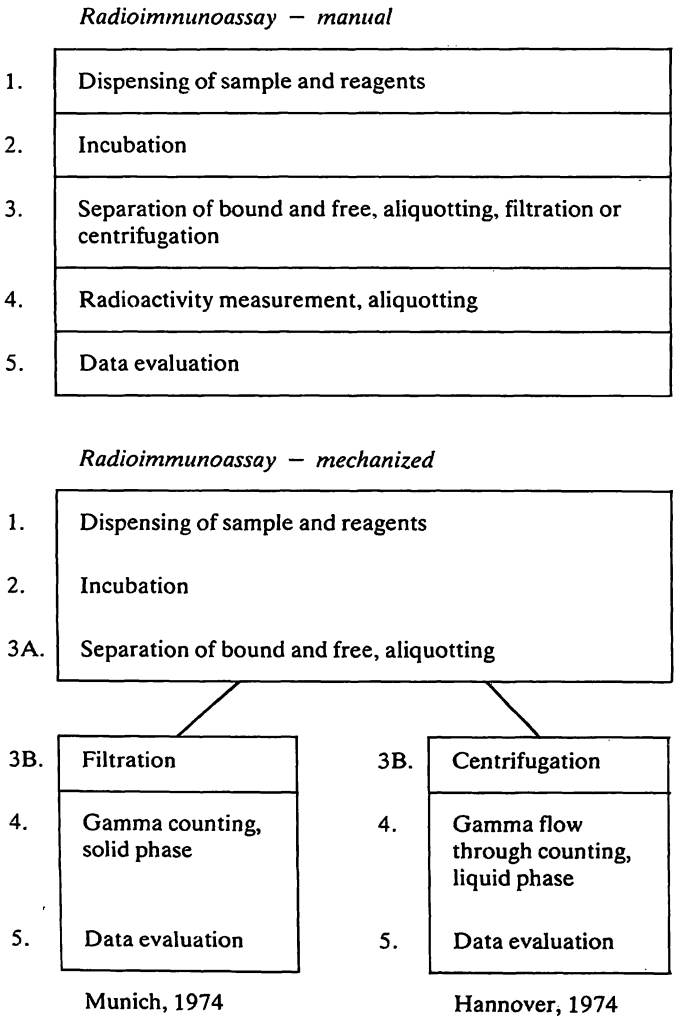


Fig. 19. Fully mechanized radioimmunoassay systems.

1. dispensing of sample and reagents,
2. incubation,
3. bound/free separation, aliquotting, filtration or centrifugation,
4. radioactivity measurement, and
5. data processing.

Amongst the developments in the early seventies, two sequentially operating systems should be mentioned. These two systems, which were characterized by an especially high degree of mechanization and flexibility, were developed by the group of *Marschner & Scriba* in Munich, and in the Department of Clinical Biochemistry in Hannover (31–36). The first three steps (see above) were integrated in a single programme-controlled unit for dispensing, dilution, sample transport and incubation. Steps 4 and 5 were also unitized. In both systems, however, the continuity of mechanization was broken by a manual phase. This is advantageous, in that it makes the process universally adaptable to a wide range of different types of radioimmunoassay. By forgoing this advantage, some radioimmunological methods, especially those with low molecular weight antigens or haptens, can be completely automated or mechanized, i.e. performed with no manual intervention between sample delivery and production of the results. For this purpose, five systems are now available (fig. 20).

Trends in Development

Trends in the future development of radioimmunoassay can be discerned with the aid of various criteria, which serve as markers for the direction and extent of growth (fig. 21). The publication peak was apparently reached in 1980/81, but the number of scientific publications is not necessarily correlated with the prevailing market.

The introduction of new commercial tests, previously used only in research, can greatly influence the market, as exemplified by the hepatitis and carcinoembryonic antigen tests, and the test for thyroid stimulating hormone. Similarly, considerable growth can be expected soon in the field of tumour markers. An expansion of the number of measured gastrointestinal hormones can also be predicted; this will occur slowly but inevitably, as the range of the available radioimmunoassays is increased. The best known example of the application of available tests in a new area is the use of the assay for thyroid stimulating hormone in neonatal hypothyreosis screening. The assays for α-foetoprotein, human chorionic somatomammotropin, ferritin, calcitonin, antidiuretic hormone and coeruloplasmin are prominent examples

Name	Manufacturer	Features	Tests
ARIA	Becton-Dickinson	Chamber with solid phase antibody, regenerable	Triiodothyronine, thyroxin, oestriol, cortisol, digoxin, thyrotropin, B_{12}/folate
Automated RIA	Technicon	Continual flow system, air bubble principle, antibody on iron particles, magnetic separation	Triiodothyronine, thyroxin, cortisol, digoxin, human placenta lactogen
Centria	Union Carbide	Batch principle (manual), centrifugal analyser, bound/free separation by ion exchange	Triiodothyronine, thyroxin, thyrotropin, insulin, digoxin, cortisol, B_{12}/folate, human placenta lactogen, lutropin, follitropin, ferritin, prolactin
Concept 4	Micromedic	Antibody-coated vessels, washing stage, 10 place racks	Triiodothyronine, thyroxin, thyrotropin, insulin, cortisol, B_{12}
Gamma flow	Squibb	Continuous flow system, air bubble principle, bound/free separation with regenerable ion exchanger	Triiodothyronine, thyroxin, digoxin, cortisol, oestriol
Kemtek 3000	Bagshawe-Kemble	15 place racks, bound/free separation by filtration, measurement of filter	Carcinoembryonic antigen, alpha-foeto-protein, human chorionic gonadotropin, human placenta lactogen, human somato-tropin, triiodothyronine, thyroxin, cortisol, oestriol

Fig. 20. Automated radioimmunoassay systems.

of the use of existing tests for tumour markers. It can also be anticipated that the development of tests for newly discovered tumour antigens, such as tissue polypeptide antigen, colon-specific antigen p and pro-adrenocorticotropic hormone, will lead to an increase in the use of radioimmunological tests in the field of tumour markers. An example of market regulation by legislation is seen in the situation that earlier resulted in Abbot's monopoly of the hepatitis test. Infectious diseases represent a possible new field of application for new tests, as evidenced by the recent development of a series of radioimmunological tests in bacteriology, parasitology and virology (tuberculosis, malaria, hepatitis). Over the last few years, simplified versions of tests have increased their share of a growing market, e.g. preprepared reaction vessels containing appropriate quantities of separately lyophilized antibody and/or labelled antigen, or premeasured quantities of labelled antigen in vessels coated with antibody. In this area, however, one can expect little further impetus for expansion, with similar low growth rates for simplified systems and their competitors, and little further development of mechanized and automated systems. It is not yet possible to foresee whether the use of homogeneous assays will result in a shift of emphasis in the market. The turnover and share of radioimmunoassays in the overall immunological analytical sector of the market will probably stabilize in the next 3–4 years. Depending on the demand for increased sensitivity, new immunological tests, especially for small antigens, will be developed as alternatives to other methods. Some existing radioimmunological tests, especially those concerned with thyroid function and drug monitoring, have been and are being replaced by non-radioimmunoassay methods. With the spread of presymptomatic diagnostic screening methods, the use of the available automated systems will increase rather than stagnate; this will perhaps lead to an improvement in the most notable weakness of radioimmunological methods, i.e. the comparability of results, which is so essential for positive clinical diagnosis (37).

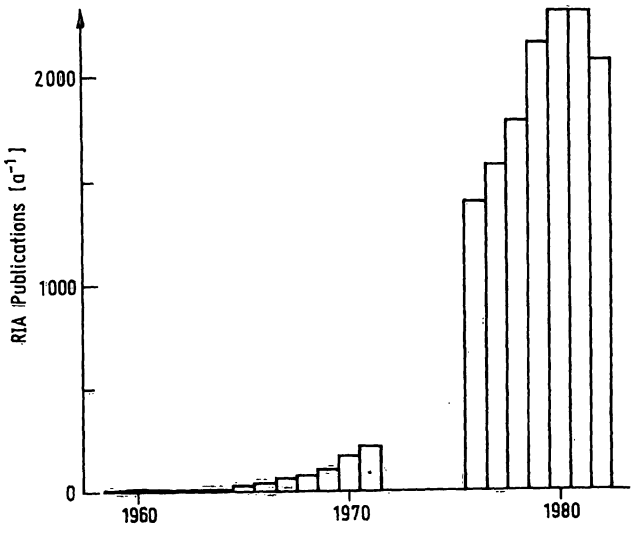


Fig. 21. Growth in the number of publication concerned with radioimmunoassay.
Growth criteria for radioimmunoassay.
New radioimmunological tests
New areas of application
Simplification of tests
Legislation
Automation
Competition
New technologies

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Enzyme-Immunoassay: A Review[1])

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Summary: The main principles of heterogeneous and homogeneous enzyme-immunoassays are reviewed. Furthermore, an overview is given of enzymes currently used as labels and methods applied for conjugate preparation. The reliability and practicability of enzyme-immunoassays are discussed. Special consideration is given to possible interferences, the detection limits and the mechanization of these assays. A range of curve-fitting methods for evaluation of the results is listed. Future prospects for the further development of enzyme-immunoassay and application of this technique are discussed.

Enzym-Immunoassay: Eine Übersicht

Zusammenfassung: Es wird eine Übersicht über die wichtigsten Prinzipien des heterogenen und homogenen Enzymimmunotests gegeben. Ferner wird über die gegenwärtig zur Markierung verwendeten Enzyme und Methoden zur Herstellung von Konjugaten berichtet. Erfahrungen hinsichtlich der Zuverlässigkeit und Praktikabilität von Enzymimmunotests werden mitgeteilt. Besondere Berücksichtigung finden die möglichen Interferenzen, die Nachweisgrenzen und Verfahren zur Mechanisierung dieser Tests. Eine Anzahl von mathematischen Methoden zur Ermittlung der Ergebniswerte ist aufgeführt. Zukünftige Aspekte der Weiterentwicklung des Enzymimmunotests und der Anwendung dieser Technik werden diskutiert.

Introduction

In recent years numerous non-isotopic immunoassays have been developed, and many different labels have been evaluated for their practicability (tab. 1). Among these new techniques the enzyme immunoassay in particular has proved to be an especially suitable alternative to radioimmunoassay. In these assays, enzymes, coenzymes, fluorigenic substrates and enzyme inhibitors are used as markers. There are several reviews dealing with enzyme-immunoassay (1–8). Enzyme-immunoassays can be categorized as either "heterogeneous" or "homogeneous".

Tab. 1. Labels used in non-isotopic immunoassays (from l.c. (1)).

Enzymes	Bacteriophage
Coenzymes	Latexes
Fluorigenic substrates	Liposomes
Enzyme inhibitors	Erythrocytes
Fluorescent dyes	Metal atoms
Fluorescence quenchers	Metal sols
Chemiluminescence precursors	Stable free radicals

Principles of Heterogeneous Enzyme-Immunoassays

Assay systems, in which the enzymic activity of the conjugate is not influenced by the antigen-antibody-reaction require a separation step and are called heterogeneous enzyme-immunoassays.

Owing to the large size and other properties of the enzyme label relative to the antigen or antibody, the systems used for the separation of free and bound labelled molecules are mostly solid-phase. For example, antibody-coated polystyrene tubes, cellulose and also magnetic polyacrylamide agarose particles have been used for the separation of bound and free enzyme conjugate (3, 4, 9–11). A separation can also be effected by precipitation of the immune complex formed with polyethylene glycol (12) or a second antibody (13) and by using a preprecipitated complex of first and second antibodies (14).

If the second antibody used for the separation step is bound to a solid phase (Fig. 1.1.2), the procedure is

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

called a double antibody solid phase (DASP) technique.

The competitive enzyme-immunoassay (fig. 1.1.1) is analogous to the well known classical radioimmunoassay of *Yalow & Berson* (15). Labelled (E-L) and unlabelled antigens (L) compete for the binding sites of a limited amount of antibody (AB). The saturation of the antibody occurs simultaneously, providing all reactants are incubated together. The principle of "sequential saturation" is preferred under certain conditions, e.g. if antigens with very low serum concentrations are to be determined (16). However, according to *Pratt* et al. this technique leads to reduced specificity (17).

Recently a new approach to heterogeneous enzyme-immunoassays has been described (18), in which the enzyme label is not only covalently linked to a ligand of the type to be determined, but also to a "tag" molecule (fig. 1.1.3). Free ligand and enzyme conjugate compete for an antibody. If the enzyme conjugate is bound to the antibody its tag is masked so that it can no longer bind to the insolubilized receptor (RI). The amount of enzyme conjugate bound to this receptor is proportional to that of the free ligand to be assayed. This procedure is called "antibody masking enzyme tag immunoassay (AMETIA). In a model system biotin has been used as the tag and avidin as the insoluble receptor (18).

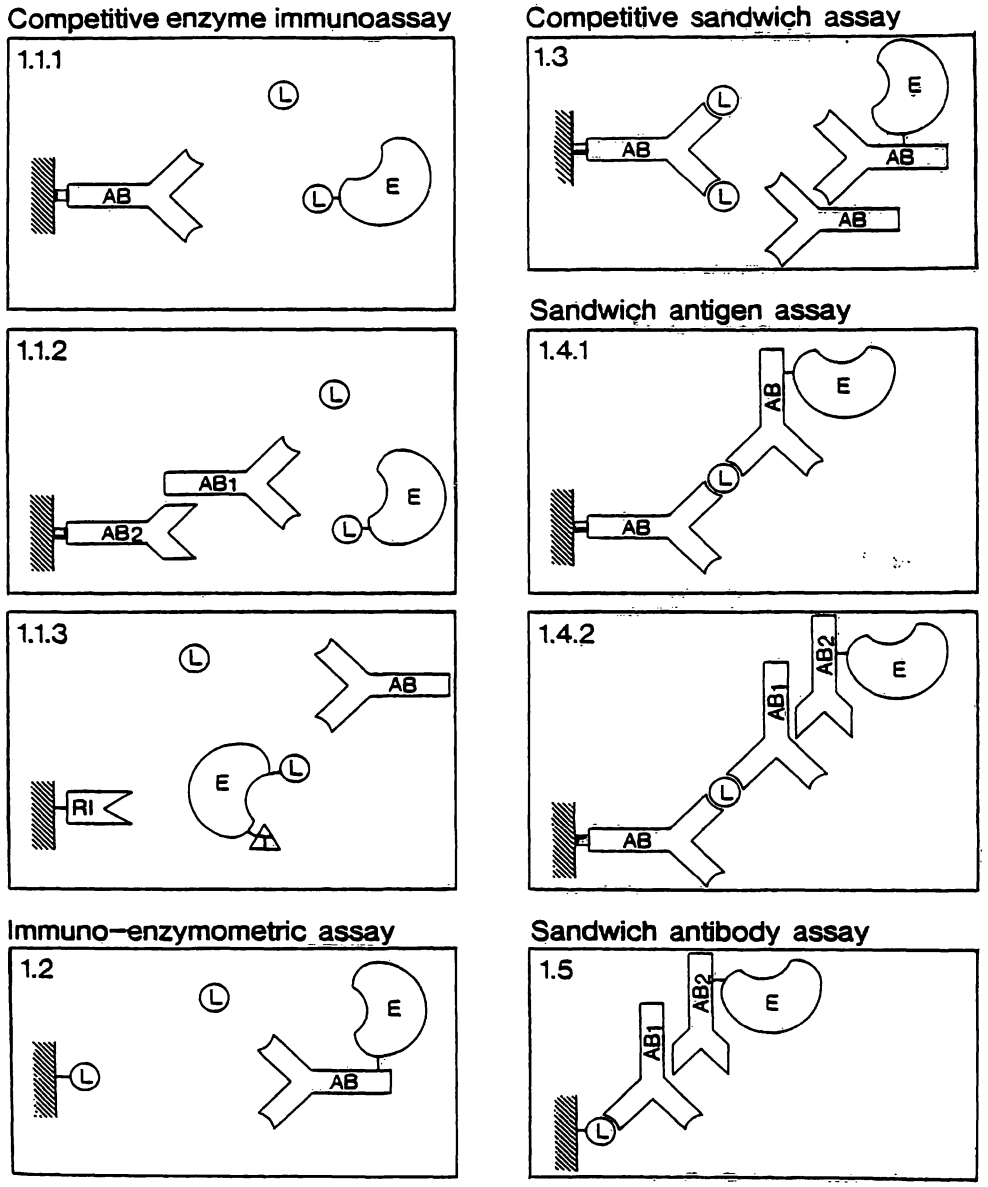


Fig. 1. Principles of heterogeneous enzyme-immunoassays (ELISA).
L ligand; E-L enzyme labelled ligand; L-E-T tagged enzyme-ligand conjugate; E-AB, E-AB_2 enzyme labelled antibodies; AB, AB_1 antibodies against ligand under test (1.1–1.4) or antibody to be determined (1.5); AB_2 antibody directed against the immunoglobulin of the species, which was immunized with the antigen to be determined (1.1–1.4) or against the antibody to be detected (1.5); RI insolubilized receptor (from l.c. (1)).

Procedures in which the sample antigen (L) is bound to an enzyme-labelled antibody (E-AB) added in excess, are classified as immuno-enzymometric assays (fig. 1.2). The remaining, free labelled antibody is separated in a subsequent step by binding with antigen (L) coupled to a solid phase, which is added in excess.

In a rapid one step procedure called inhibition enzyme-immunoassay the antigen-antibody interaction between solid phase-coupled antigen and an enzyme-labelled antibody is inhibited by free antigen of the sample. The enzymic activity detected on the solid phase is inversely proportional to the amount of antigen present in the sample.

"Sandwich" assays have been described in numerous modifications (19). In a variation of the sandwich antigen assay (fig. 1.4.1), the bound antibody (AB_1) is indirectly labelled by a second enzyme-labelled antibody (E-AB_2), which is directed against the first (AB_1) and should not react with the antibody (AB) attached to the solid phase (fig. 1.4.2). The antibodies AB_1 and AB should be be obtained from different species. Such an indirect labelling can also be applied to immuno-enzymometric tests (5) and inhibition enzyme-immunoassays (20). The method of indirect labelling has the advantage that the same enzyme-antibody conjugate can be used in assays for various different antigens. Thus the often difficult direct labelling of the antibody may be circumvented. In the sandwich antibody assay (fig. 1.5), the antibody to be determined (AB_1) reacts with an antigen (L) bound to the solid phase, and is detected by a second enzyme-labelled antibody (E-AB_2).

Enzyme-Labels Used in Heterogeneous Enzyme-Immunoassays

The quality of an enzyme-immunoassay depends very much on the purity of the antigen or hapten used for immunization, calibration and conjugation, the specificity of the antibody and the choice of a suitable enzyme label. Sensitive assays require a highly purified enzyme with a high turnover number, and a low detection limit for the reaction product (4).

The enzymes listed in table 2 have proved generally useful. The enzymes most frequently used so far for heterogeneous enzyme-immunoassays are horseradish peroxidase, alkaline phosphatase and β-*D*-galactosidase.

The determination of the enzyme activity is chiefly performed by photometry. In order to increase the detectability of the assays, however, fluorigenic, radioactive and chemiluminescence-producing substrates are also used. In a further procedure, the activity of peroxidase bound to an antibody-coated membrane is determined by an iodide-sensitive elec-

Tab. 2. Enzyme labels used in heterogeneous enzyme-immunoassays (from l.c. (1, 21)).

Enzyme	Source	Indicator	Analytical principle
Peroxidase (EC 1.11.1.7)	Horse-radish	H_2O_2/chromogen	Photometry
		H_2O_2/pyrogallol or luminol, photon	Luminometry
		H_2O_2/KI/ΔE [mV]	Potentiometry
Alkaline phosphatase (EC 3.1.3.1)	Calf intestine	4-Nitrophenol	Photometry
	E. coli	4-Methylumbelliferone	Fluorimetry
β-*D*-Galactosidase (EC 3.2.1.23)	*E. coli*	2-Nitrophenol	Photometry
		4-Methylumbelliferone	Fluorimetry
Glucoamylase (EC 3.2.1.3)	*Rhizopus niveus*	Glucose/NADPH	Fluorimetry
Glucose oxidase (EC 1.1.3.4)	*Aspergillus niger*	H_2O_2/chromogen	Photometry
Acetylcholinesterase (EC 3.1.1.7)	*Electrophorus electricus*	[^{3}H]Acetylcholine	Scintillation spectrometry
Glutamate decarboxylase (EC 4.1.1.15)	*E. coli*	$^{14}CO_2$	Scintillation spectrometry
Catalase (EC 1.11.1.6)	Beef liver	H_2O_2/enthalpy	Calorimetry
Urease (EC 3.5.15)	Jack bean	NH_3/bromocresol purple	Photometry
	Bacillus pasteurii		

trode (tab. 2). A thermometric enzyme-immunoassay with adequate sensitivity has been described, in which the enthalpy of the enzymic reaction is measured (22). In this assay, a continuous flow system with immobilized antibodies is used.

Coupling procedures

Enzymes can be coupled to antibodies and protein antigens by various procedures, using e.g. glutaraldehyde, periodate or N,N'-*o*-phenylenedimaleimide. Hapten-enzyme conjugates can be formed by the carbodiimide and mixed anhydride methods. The more important of these procedures have recently been reviewed (6).

Suitable coupling reactions should allow a high degree of incorporation of enzyme into the conjugate with minimal changes in immunoreactivity and in the properties of the coupled enzyme. Furthermore the degree of substitution of the enzyme by the hapten must be adequate and conjugates should be stable.

The immunoreactivity of a compound can be altered by cross-linking reactions. If, for instance, the enzyme is coupled to important antigenic sites on the hapten, a reduced avidity of the antibody for the labelled compound may result (6). On the other hand, the affinity of the labelled antigen for the antibody may be higher than that of the free antigen, if an antigen derivative similar to the immunogen is used to form the labelled antigen (23). In these cases antibodies may recognize the bridge between antigen and carrier molecule in addition to the antigen itself. Furthermore, multivalent labelled antigens may be able to compete very effectively with unlabelled antigen for antibody binding sites. The combination of bivalent antibody with multivalent antigen, especially, may yield extremely high overall affinities (23). If the affinity of the antibody for labelled antigen is much lower or higher than that for unlabelled antigen, a loss in sensitivity of the assay may result (6, 23).

The problem of the degree of substitution of the enzyme by the hapten requires further systematic investigation. At present, a convenient approach is to take conjugates with varying degrees of hapten substitution, and select that which provides the best standard curve (6).

Principles of Homogeneous Enzyme-Immunoassays

In homogeneous enzyme-immunoassays a separation step is avoided. The enzymatic activity is influenced by the antigen-antibody reaction in these tests.

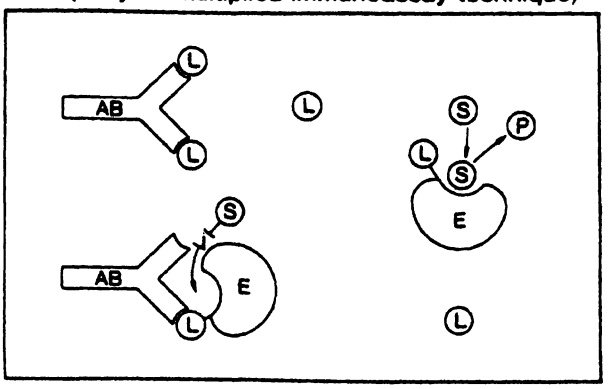
In the two variations of the EMIT (fig. 2.1.1, 2.1.2) the activity of the marker enzyme is inhibited or increased, if the conjugate is bound to the antibody (24, 25).

The "enzyme channeling immunoassay" (ECIA) represents a rather complex assay system (fig. 2.2). In this procedure (8) the ligand (L) is covalently labelled with an enzyme (E_1). This conjugate competes with the ligand of the sample for the binding sites of a limited amount of antibody which is co-immobilized with the second enzyme (E_2) on beads (B). The overall enhancement in the rate of formation of the reaction product (P_2) from the substrate (S) depends on the ligand (L) concentration. In the presence of a high ligand (L) concentration there would be less ligand-enzyme conjugate (E_1-L) bound to the antibody and consequently less substrate (S) converted to the product (P_2). In order to reduce background reactions, the product P_1 generated by the unbound conjugate is converted to the product Q by a further soluble, scavenger enzyme (E_3). Recently an enzyme channeling immunoassay for determination of IgG has been described in which hexokinase-labelled antibody and glucose-6-phosphate dehydrogenase-labelled antigen were used (26).

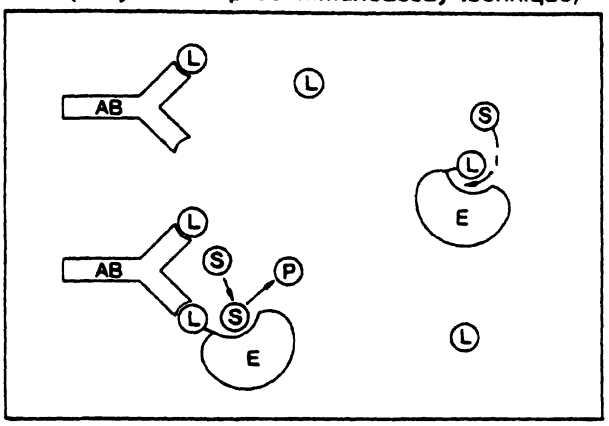
A further homogeneous enzyme-immunoassay (enzyme-enhancement immunoassay, EEIA) for proteins has been described (27), which avoids the need for a labelled antigen (fig. 2.3). The enzyme-labelled antibody (E-AB_1) and the succinylated antibody (AB_2) form an immune complex with a polyvalent antigenic analyte (L) present in the sample. An enzyme within this negatively charged micro-environment converts a substrate (S) to a product (P_2) which forms a second light-scattering phase. The product (P_1) formed by the free enzyme-conjugate remains soluble. The concentration of the second-phase product (P_2) is proportional to the amount of sample antigen (L) and can be determined by turbidimetry.

Fig. 2. Principles of homogeneous enzyme-immunoassays. All reactants are present in one reaction medium: L ligand; E-L, E_1-L enzyme labelled ligands; M-L ligand-substituted enzyme modulator; FAD-L ligand covalently linked to flavin adenine dinucleotide; S-L ligand labelled substrate; P-L ligand labelled product; AB limited amount of antibody; AB_1 antibody to be detected; AB_2 succinylated antibody; AB_1-E, AB_2-E enzyme labelled antibodies; E_2-B-AB antibody co-immobilized with an enzyme (E_2) on fine beads (B); E, E_1, E_2, E_3 enzymes; Apo-E apoenzyme; Holo-E holoenzyme; S substrate; RBC erythrocyte; P, P_1, P_2, Q Products of enzymic reactions, i inhibition of the "marker enzyme" (from l.c. (1)).

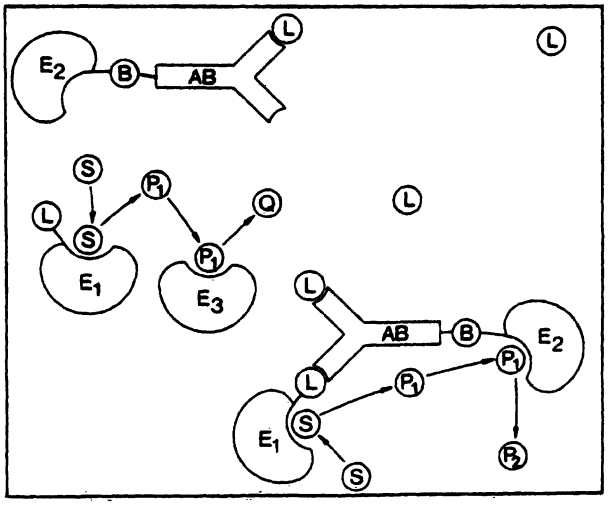
2.1.1
EMIT (enzyme multiplied immunoassay technique)



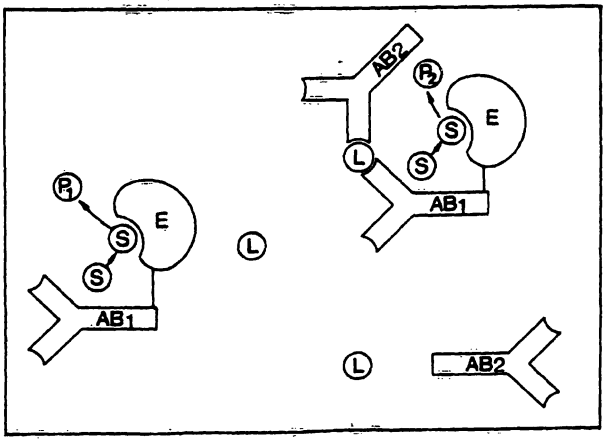
2.1.2
EMIT (enzyme multiplied immunoassay technique)



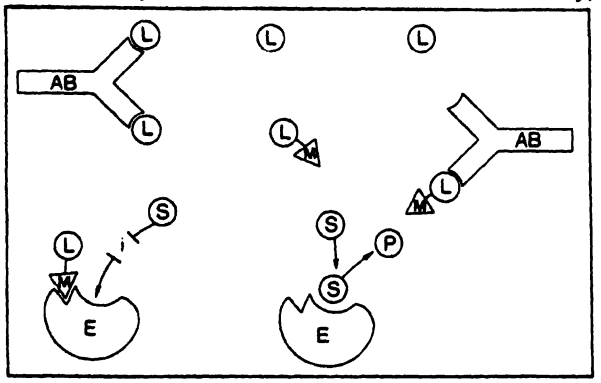
2.2 ECIA (enzyme channeling immunoassay)



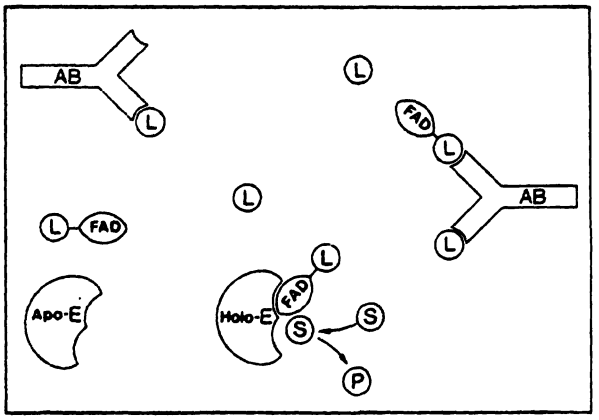
2.3 EEIA (enzyme–enhancement immunoassay)



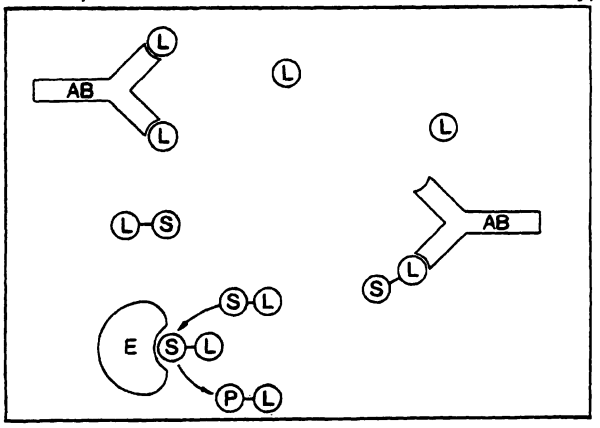
2.4
EMMIA(enzyme modulator mediated immunoassay)



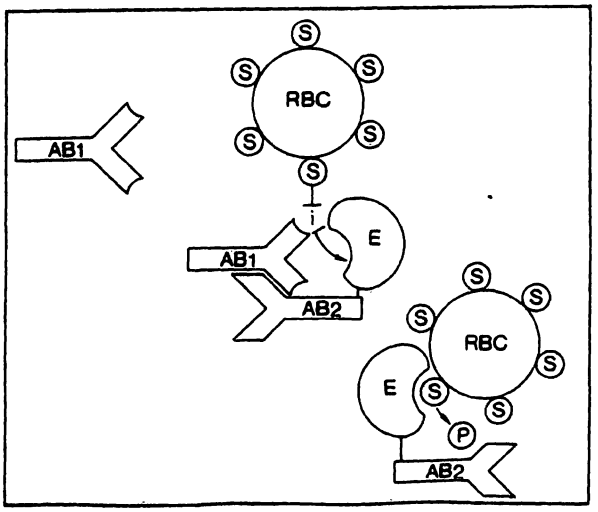
2.5 PGLIA (prosthetic–group–label immunoassay)



2.6
SLFIA(substrate–labeled fluorescent immunoassay)



2.7 EIIA (enzyme inhibition immunoassay)



The enzyme modulator mediated immunoassay (EMMIA) described by *Ngo* et al. (28) is based on the ability of a ligand-coupled enzyme modulator (L-M) to influence the activity of the indicator enzyme (fig. 2.4). Ligand-substituted enzyme modulator (L-M) and ligand from the sample compete for a limited amount of antibody (AB). If the enzyme modulator (L-M) is bound to the antibody, it cannot affect the activity of the indicator enzyme. Modulators which increase or decrease the enzyme activity can be used. In assays with an inhibiting modulator the enzyme activity is inversely proportional to the concentration of the analyte. This technique has been used for determination of thyroxine (29).

A further homogeneous enzyme-immunoassay is based on a ligand-labelled enzyme prosthetic group (30). In the scheme of figure 2.5, ligand-flavin-adenine-dinucleotide conjugate (L-FAD) and ligand (L) from the sample compete for a limited amount of antibody (AB). If the ligand-FAD conjugate is bound by the antibody, it can no longer combine with the apoenzyme (Apo-E) to form an enzymatically active holoenzyme (Holo-E). Thus the observed enzyme activity is directly related to the concentration of the analyte (L). This principle can be used for the determination of haptens and antigens (8, 30, 31).

The principle of the substrate-labelled fluorescent immunoassay is schematically presented in figure 2.6. In this assay a ligand (L) labelled with a fluorigenic substrate (S) is used. This conjugate is non-fluorescent and competes with the analyte (L) for a limited amount of antibody (AB). When the substrate-ligand conjugate (S-L) is bound by the antibody, it cannot be converted by an enzyme to a fluorescent product (P-L). The amount of conjugate available for reaction with the enzyme and the resulting fluorescence intensity are proportional to the concentration of the analyte. Since in assays of this type the enzyme does not produce an amplification effect and the amount of unbound substrate-labelled ligand available for the enzyme depends on the analyte concentration, it is necessary to use a fluorigenic substrate to label the ligand. Substrate-labelled fluorescent immunoassays have been described for the determination of haptens and antigens (32, 33).

A further homogeneous immunoassay has been described by *Wei* et al. (34). A hypothetical scheme of this system is shown in figure 2.7. Rabbit antihuman IgG (AB_2) was labelled with phospholipase C (E). The enzymatic activity of the conjugate was inhibited by human IgG (AB_1). The substrates (S) used were phospholipids, which are components of the erythrocyte membranes and therefore may be viewed as being immobilized. When IgG (AB_1) forms a complex with the enzyme-anti-IgG conjugate, it sterically prevents an interaction between the enzyme label (E) and the substrate (S).

Other competitive procedures, in which coenzymes are used to label the ligand (35, 36), may also be classified as homogeneous enzyme-immunoassays. If the ligand-coenzyme conjugate is bound to an antibody, its cycling in a suitable enzymic cycle is proportionally reduced. This inhibition is reversed by unconjugated ligands in competitive binding reactions. So far, however, this assay principle has gained no significance in clinical diagnosis.

Enzyme-Labels Used in Homogeneous Enzyme Immunoassays

The enzymes listed in table 3 have proved generally useful as labels in homogeneous enzyme-immunoassays.

In the EMIT, an NAD-dependent glucose-6-phosphate dehydrogenase is mainly used. The activity of this enzyme can be easily determined photometrically or fluorimetrically. In an antigen-labelled homogeneous enzyme-inhibition immunoassay for serum proteins and an enzyme-enhancement immunoassay, β-galactosidase was used as label and a synthetic macromolecular dextran-linked *o*-nitrophenyl-β-galactoside as substrate (27, 37). *Litman* et al. (26) employed the enzyme pair, hexokinase and glucose-6-phosphate dehydrogenase, in an enzyme channeling immunoassay. Scavenging was achieved in this assay by phosphoglucose isomerase (EC 5.3.1.9) and phosphofructokinase (EC 2.7.1.11).

In various other homogeneous enzyme-immunoassays the indicator enzyme is coupled neither to the antigen nor to antibody (fig. 2). Horseradish peroxidase (EC 1.11.1.7) or acetylcholinesterase (acetylcholine hydrolase; EC 3.1.1.7) have been used in enzyme modulator immunoassays (28, 29). In prosthetic-group-label homogeneous immunoassays flavin adenine dinucleotide has been used as the prosthetic group and glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* as the holoenzyme (30, 31). For homogeneous substrate-labelled fluorescent immunoassays β-galactosidase (β-*D*-galactosidase galactohydrolase, EC 3.2.1.23) from *Escherichia coli* and a fluorigenic substrate (e.g. β-galactosyl-umbelliferone) was employed (32, 33).

Tab. 3. Enzyme labels used in homogeneous enzyme-immunoassays (from l.c. (1)).

Enzyme	Source	Indicator	Analytical principle
Lysozyme (EC 3.2.1.17)	Chicken egg-white	Cell wall fragments of *Micrococcus luteus*	Turbidimetry
Malate dehydrogenase (EC 1.1.1.37)	Pig-heart mitochondria	NADH	Photometry
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	*Leuconostoc mesenteroides*	NADH	Photometry Fluorimetry
Hexokinase (EC 2.7.1.1)	Yeast	*	*
β-*D*-Galactosidase (EC 3.2.1.23)	*E. coli*	Dextran-linked 2-nitrophenol **	Photometry Turbidimetry
β-Amylase (EC 3.2.1.2)	Potato	Starch/reducing groups	Photometry
Phospholipase C (EC 3.1.4.3)	*Clostridium welchii*	Haemoglobin release	Photometry

* Antibody-hexokinase conjugates were used in combination with glucose-6-phosphate dehydrogenase (26).
** "Second-phase product" not further described by the authors (27).

Enzyme-hapten conjugates have been prepared, for example, by mixed anhydride reactions or by acetimidate-linking (38, 39), and enzyme-antibody conjugates (26, 27) by the *m*-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) method (4).

Application of Enzyme-Immunoassays

The field of application of the enzyme-immunoassay corresponds largely to that of radioimmunoassay. Assays for the determination of plasma proteins, tumour antigens, drugs, hormones, antigens of pathogenic organisms and antibodies have been developed (4, 6, 19).

The imprecision of enzyme-immunoassays is of the same order of magnitude as that of corresponding radioimmunoassays. With partially or fully mechanized enzyme-immunoassays for quantitative determinations, between-days coefficients of variation of about 2–10% were achieved in the medium working range of these tests (2).

The inaccuracy of enzyme-immunoassays has been examined in many comparative studies. Mainly comparable results were observed with enzyme-immunoassays and other immunological and non-immunological procedures (2).

Interference

Enzyme-immunoassays may be subject to interference due to disturbances of the enzymic or immunological reaction. Endogenous enzymes with biological effects, similar to those of the enzyme used as label, or other factors which directly influence the enzymic reaction, may lead to wrong results. Furthermore, interference can be caused by an unspecific binding of the conjugate to the solid phase, or by certain plasma proteins or transport proteins which compete with the antibody. In the substrate-labeled fluorescent immunoassay, interference occurred in a few cases, presumably due to the presence of fluorescing compounds in the sample (42).

With the ELISA-technique, interference by light absorbing substances such as haemoglobin or bilirubin or by turbidity, or by endogenous enzymes or enzyme inhibitors, is reduced if the activity of the bound enzyme conjugate is measured. However, the washing steps required with this technique may theoretically influence the imprecision of these assays considerably.

In homogeneous enzyme-immunoassays for quantitative determinations, interference is only rarely observed. An essential reason for this appears to be the high dilution of the serum sample in the reaction medium. To avoid interference from certain serum proteins in some assays the serum must be pretreated (2).

Cross-reactions with other compounds occur if the antibody lacks specificity. The antibodies of various commercial assays for quantitative determinations show a relatively high specificity (2). However, considerable differences in specificity have been observed, for example, among various commercial immunoassays for determination of theophylline (42).

Detection Limits

In a number of heterogeneous enzyme immunoassays the detection limits are the same as those of radioimmunoassay (2). In general, the detection limits of radioimmunoassays range from 1—500 pmol/l or 0.2—50 fmol/tube (4). Moreover heterogeneous enzyme immunoassays with an extraordinarily high detectability have been described. Such assays were capable of detecting even 1 amol/tube (10^{-18} mol) of ornithine-δ-aminotransferase (43) or 24000 molecules of purified mouse myeloma IgG (44).

The detectability of homogeneous enzyme-immunoassays is lower than that of heterogeneous tests (2). The detection limits and the lower limits of the working range with EMIT for various drugs and thyroxine were 10^2—10^7 pmol/l (2), with substrate-labelled fluorescent immunoassays for several drugs and immunoglobulins the corresponding range was 10^5—10^6 pmol/l (1, 32—33), and with the enzyme modulator mediated immunoassay for thyroxine, 6.4×10^3 pmol/l (29). However, the detectability of these assays is sufficient for the determination of many diagnostically relevant compounds (2).

Evaluation of the results

For the evaluation of the results from enzyme-immunoassays computers are increasingly used. Manual procedures are lengthy and sometimes less dependable.

A range of available curve-fitting methods is shown in table 4. Some of these procedures are based on certain models, whereas "data-based" methods can be applied without knowledge of the type of function underlying the calibration curve. The most suitable methods must first be ascertained for each enzyme-immunoassay.

Tab. 4. Curve fitting methods for enzyme-immunoassay (from l.c. (2)).

1.	Model-based methods
1.1	Parabolic regression
1.2	Linear regression after logit-log transformation
1.3	Weighted linear regression after logit-log transformation
1.4	Weighted non-linear regression after logit-log transformation
2.	Data-based methods
2.1	Manual curve-fit
2.2	Polygonal interpolation
2.3	Empiric spline-interpolation
2.4	Cubic spline-interpolation
2.5	Spline approximation

Future Aspects

In the past few years enzyme-immunoassays have been increasingly used, especially for the determination of substances like drugs, hormones, antigens of pathogenic organisms and antibodies.

The development of enzyme-immunoassay is currently still in a state of flux. Further possible sources of improvement lie in a better standardization of the reagents and methods, in the search of more effective marker enzymes and better cross-linking reagents. Systematic investigations are needed concerning the influence of the degree of labelling, the site of cross linking and the nature of the bridge on the performance of an assay. Alternative procedures for the determination of enzyme activities, such as fluorimetry and calorimetry should be evaluated.

The use of suitable monoclonal antibodies could contribute to an improvement of the specificity of enzyme-immunoassays. Although it is still quite difficult to obtain suitable high-affinity monoclonal antibodies, the application of such antibodies appears to be promising in the immunodiagnostic field. For example, in a newly developed enzyme immunoassay for determination of thyrotropin, which is based on the "sandwich" principle, monoclonal anti-β-thyrotropin antibodies and peroxidase labeled Fab' fragments of sheep antibody have been used (41). The results obtained by this method were in relatively good agreement with those determined by radioimmunoassay (tab. 5). The use of selected pairs of monoclonal antibodies could allow the development of new heterogeneous and homogeneous assay principles.

Mechanization

A far-reaching mechanization of enzyme-immunoassays appears to be essential. According to existing experience, the improvement of the reliability and practicability of these assays by mechanization is likely to be far greater than the beneficial effects of mechanization on the commonly used clinical chemical routine procedures. As enzyme-immunoassays show non-linear calibration curves, the accuracy of these tests depends very much on the precision, which can be considerably increased by mechanization.

Tab. 5. Comparison of the results obtained by enzyme-immunoassay (ELISA, Enzymun-Test® TSH) and radioimmunoassay (TSH-Henning) for determination of thyrotropin (TSH) in serum samples from patients.

Methods			n[1])	Standardized principal component		$s_{y,x}$[2])	$\bar{y}$(s)[3])	$\bar{x}$(s)	t[4])	Correlation coefficient
(y)	vs	(x)		Slope	Intercept					
ELISA	vs	RIA	40	0.90	−0.30	1.81	11.5 (12.1)	13.0 (13.4)	3.22*	0.97

[1]) number of contributing values
[2]) standard error of the residuals
[3]) mean value (mU/l) with standard deviation in parenthesis
[4]) t-value (paired t-test)
* significance of the bias $\bar{x} - \bar{y}$($p < 0.05$). Three samples were excluded from statistical evaluation of the results:
1. ELISA: <0.5 mU/l, RIA: 1.5 mU/l;
2. ELISA: <0.5 mU/l, RIA: 1.7 mU/l;
3. ELISA: <0.5 mU/l, RIA: 1.1 mU/l. (l.c. (40))

Furthermore, the costs of reagents and technician time can be considerably reduced if suitable mechanization is chosen (2). A large reduction in reagent costs may be achieved by adaptation of EMIT to centrifugal analysers, and of the substrate-labelled fluorescent immunoassay to the Micro-ERMA (Micro Enzyme Reaction, Multiple Assay) system (45).

Because of the incubation periods, and the washing- and separation steps, it is difficult to fully to mechanize the ELISA-technique. Certain steps, such as the washing procedure, the dispensation of the reagents and the photometric measurement have already been mechanized (tab. 6).

The performance of heterogeneous enzyme-immunoassays generally takes several hours at least. In our experience 200—300 patient samples can be assayed for thyroxine or hepatitis B surface antigen by one technical assistant per day using partially mechanized systems. Up to 4000 samples have been analysed per day with an enzyme-immunoassay for determination of antibodies against *Trichinella spiralis* (46).

Tab. 6. Various analytical systems used for enzyme immunoassays.

Homogeneous enzyme-immunoassays	Heterogeneous enzyme-immunoassays
Centrifugal analysers (i.e. Cobas Bio)	ELISA-Meßplatz Eppendorf
AutoLab 5000	Abbott Quantum I
ACA Du Pont	Riele PMC Automatik
Eppendorf ACP 5040	Gilford EIA-PR 50
ABA-100	LKB 2074
Fluorostat	Titertek-Multiskan
Optimate	Enzymun-Test® System ES 22

The mechanization of heterogeneous enzyme-immunoassays may perphaps be facilitated in the future by the use of aqueous two-phase systems for separation of bound and free ligand. The principle is that free and bound antigen are distributed unevenly between two immiscible water-soluble phases and can subsequently be recovered from separate phases (47).

Homogeneous enzyme immunoassays have been partially and fully mechanized by use of many different analytical systems (2). Several examples are given in table 6. Single emergency determinations of drugs such as theophylline, can be performed by EMIT within 10—20 minutes. By using quantitative EMIT drug assays adapted to an Eppendorf system ACP 5040, for example, about 250 patient samples can easily be analysed by one technical assistant per day (2). With the ACA from Du Pont, calibration curves for EMIT were stable for many weeks, provided that the same lot of reagents was used (48).

The development of dry chemistry systems could greatly simplify the performance of enzyme-immunoassays. In the EMIT®-st™ drug detection system all the reactive components such as enzyme conjugate, substrate, antibody and buffer are already present in one vial in the dry form. The assay is carried out merely by adding patient sample plus water and taking a reading at a fixed time. Recently, reagent strip systems for theophylline and phenytoin have been described which are based on the prosthetic-group-label homogeneous immunoassay (31). Such reagent strip systems may allow rapid determinations and evaluation of the results either with the unaided eye or by use of reflectance spectrophotometry. Further developments in this field might include dry, solid-phase reagent systems such as multilayer film elements (49).

Future fields of application

The demand for existing and new immunoassays is expected to grow considerably in future. The determination of numerous clinically relevant proteins and drugs in serum or plasma will gain increasing importance (1). Furthermore, enzyme-immunoassays will have a wide application as screening tests for antigens from pathogenic organisms and antibodies directed towards them. Moreover such assays will be frequently used in diagnostic strategies, which are concerned with presymptomatic diagnosis. It is to be expected that the use of monocloncal antibodies in particular will lead to the discovery of many new diagnostically important antigens, which for technical reasons have so far been inaccessible. Furthermore immunoassays could also be applied in non-medical disciplines dealing with food production or environmental pollution.

It can be predicted that the further development of immunological techniques will contribute to a considerable improvement of clinical diagnosis and therapy.

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Luminescence Immunoassays: Problems and Possibilities[1])

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Summary: This article reviews the field of bioluminescence and chemiluminescence immunoassays, and gives examples from past and present work in these areas. The problems of luminescence immunoassays are discussed, and include the choice of label, solid phase, immunogen/hapten-protein conjugates for immobilisation and the stability of bio- and chemiluminescent reagents.

Examples have been given to show the stages in the development of luminescence immunoassays up to their acceptance for routine clinical in-vitro diagnostic use.

Luminometers and commercial kits have been discussed, with regard to the present situation in these fields and the personal experience with two semi-mechanized luminescence analysers in routine use.

Lumineszenz-Immunoassays: Probleme und Anwendung

Zusammenfassung: Die vorliegende Arbeit gibt einen kurzen Überblick über das Gebiet der Lumineszenz, hauptsächlich über Lumineszenzimmunassays. Ausgehend von den ersten Untersuchungen bis hin zum ak-

Abbreviations used in the text.

Diazoluminol	Diazotised 5-amino-2,3-dihydrophthalazine-1,4-dione
Diazoisoluminol	Diazotised 6-amino-2,3-dihydrophthalazine-1,4-dione
Pyruvate kinase	ATP: pyruvate phosphotransferase – EC 2.7.1.40
SPALT	Solid phase antigen luminescence technique
ILMA	Immunoluminometric assay
IRMA	Immunoradiometric assay
ILSA	Immunoluminometric labelled second-antibody assay
CELIA	Chemiluminescent immunoassay
RIA	Radioimmunoassay
EIA	Enzymeimmunoassay
B_0	Binding of the zero standard under assay conditions
B_x	Binding of a given standard x under assay conditions
UB	Unspecific (non-specific) binding
EOB	Enzyme oxidant (reagent) blank
T	Signal of the tracer given to each tube (total counts)
Buffer 4L	0.05 mol/l Tris-HCl containing 0.02 mol/l KCl and 2.5 g/l bovine serum albumin, pH 7.4
Buffer 4N	As buffer 4L, but with 0.1 g/l NaN_3
PBS	0.05 mol/l phosphate buffer containing 0.15 mol/l NaCl, pH 7.4
PBS-Tween	As PBS, with 0.5 ml/l Tween 20
Tween 20	Polyoxyethylene sorbitan monolaurate
LKB 1250	Single channel manual luminometer – LKB-Wallac, Turku, SF.
LKB 1251	25-sample semi-automatic luminometer – LKB-Wallac
LB 950	250/300 sample semiautomatic luminometer – Laboratorium Prof. Dr. Berthold, Wildbad, D.

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

tuellen Stand der Lumineszenzimmunassays wird die Entwicklung der Bio- und Chemilumineszenz aufgezeigt. Auf die praktischen Probleme bei der Entwicklung von Lumineszenzimmunassays wie Wahl des Luminogens, Herstellung und Stabilität von bio- und chemilumineszierenden Markersubstanzen, die Bedeutung der Verwendung des geeignetsten Immunogens sowie die Festphasen-Problematik wird umfassend eingegangen. Beispiele von Lumineszenzimmunassays und deren Entwicklung und Optimierung bis hin zur routinemäßigen Anwendung sind beschrieben.

Luminometer und kommerzielle Testbestecke werden kurz angesprochen, insbesondere die persönlichen Erfahrungen mit zwei teilmechanisierten Luminometern in der Routine.

Introduction

Many scientists and philosophers have dedicated part of their studies to luminescent phenomena over the past two millenia. *Gaius Plinius Secundus* described marine phosphorescence in the second century A.D. Other well-known figures who showed interest in luminescence include *Francis Bacon, Galileo Galilei, Leonardo da Vinci* and *Theophrastus Bombastus von Hohenheim* (*Paracelsus*); but it was *Robert Boyle* in the 17th century who made the first scientific observations which defined the properties of bioluminescence in his studies on rotten wood, shining flesh and stinking fish (1, 2).

The terms luciferin and luciferase were first introduced by *Dubois* in a series of articles written in the last quarter of the 19th century (3, 4).

Practical application of bio- and chemiluminescence are a phenomenon of the 20th century and have been dependent upon the progress in the electronic industry associated with photon detection devices.

Chemiluminescent compounds include lucigenin (5) acridinium derivatives (6), luminol and isoluminol (7, 8) and pyrogallol (9) and it has become possible to synthesize derivatives of these compounds for use in chemiluminescent detection systems both in-vivo and in-vitro. An in-vivo application is the study of phagocytosis (10), whereas an in-vitro application, luminescence immunoassays (11—15) forms the main part of this article, with an accent upon the problems and pitfalls encountered, especially in the laboratory of the author.

Principles of Luminescence

Chemiluminescence

Chemiluminescence occurs when excited molecules return to a stable state with the emission of energy as light quanta. The efficiency of chemiluminescent systems is usually very low (under 5%), the majority of energy being dissipated in "dark" reactions. It has been reported that specially synthesised oxamides have an efficiency of above 30% (16), and it is to be awaited that when the need arises, chemiluminescent compounds with a highly efficient light output will be synthesised.

The reactions which occur during the light-generation process appear to be of two main types, namely a one-electron transfer process resulting in radical ion-pairs, and secondly energy transfer from singlet oxygen (16, 17).

As the name chemiluminescence implies, one does not necessarily need biological agents as intermediates in the light-generation reactions, although very often a pseudoperoxidase, e.g. haemin or microperoxidase from cytochrome-c, is used in conjunction with a peroxide or persulphate or perborate (18).

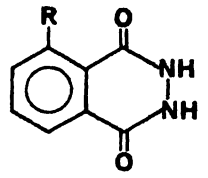
Chemiluminescence can be regarded as a "one-off" reaction, i.e. when the reaction has taken place there is no second chance of measurement, as in the case of radioactivity. This is an important and practical point to note when considering assays using a chemiluminescent label!

The main groups of compounds at present used in chemiluminescent studies are, as stated above, derivatives of luminol, isoluminol and acridine. Figure 1 shows a few chemiluminescent compounds in use, whereas table 1 shows the main characteristics of chemiluminescence as well as both its advantages and disadvantages.

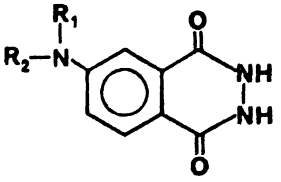
Bioluminescence

As the name implies, this type of luminescence is associated with biological systems, either in-toto or in isolated form. The components involved in the light-generating reactions include a luciferin in reduced form as substrate, together with a luciferase as specific enzyme. An energy-supplying substrate or co-factor is present, often in the form of NAD(P)H or ATP.

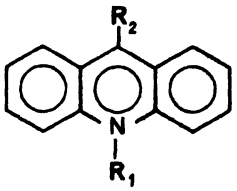
Figure 2 shows the structures of a few luciferins and table 2 lists a few luminescent organisms. The basic



Luminol derivatives
R = NH_2 – Luminol
R = N = N^+X^- – Diazoluminol



Isoluminol derivatives
R_1 and R_2 = H – Isoluminol
R_1 = C_2H_5 R_2 = $(CH_2)_2NH_2$ – 2-aminoethyl-N-ethyl isoluminol
R_1 = C_2H_5 R_2 = $(CH_2)_4NH_2$ – 4-aminobutyl-N-ethyl isoluminol
R_1 = C_2H_5 R_2 = $(CH_2)_5NH_2$ – 5-aminopentyl-N-ethyl isoluminol
R_1 = C_2H_5 R_2 = $(CH_2)_6NH_2$ – 6-aminohexyl-N-ethyl isoluminol (see l.c. (22))



Acridine derivatives
R_1 absent, R_2 = H – Acridine
R_1 = CH_3 R_2 = $COOR_3$ where R_3 is an alcohol e.g. a sterol – Acridinium ester
R_1 = CH_3 R_2 = N-methyl acridine – Lucigenin (9,9'-bis-(N-methylacridinium nitrate))

Fig. 1. Some of the most commonly used chemiluminescent compounds.

tenets of bioluminescence as stated by *Robert Boyle* (1, 2) are listed in table 3.

The most commonly used luciferin-luciferase system is that derived from the American firefly (*Photinus* sp.). The purified reagents can be purchased either separately or as a "ready-to-use" mixture, sold as ATP-monitoring reagent or a similar name by several chemical producers.

In the bioluminescent immunoassays developed in this laboratory (14) pyruvate kinase was chosen as label, using ADP and phosphoenolpyruvate as substrates, the ATP being produced being monitored kinetically in a luminometer attached to an integrator/plotter (LKB 1250).

Tab. 1. Characteristics, advantages and disadvantages of chemiluminescence.

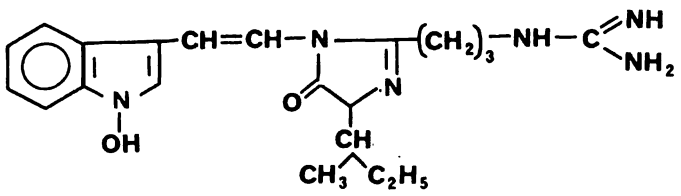
Characteristics
a. Chemiluminescence is commonly associated with "non-biological"systems.
b. It often involves compounds related to luminol, isoluminol and acridine.
c. An enzyme is not necessary for light production, although a pseudoperoxidase is often used to generate nascent oxygen.
d. Active oxygen is needed (O_2^- or O_2H^-) to initiate chemiluminescence.
Advantages
a. All molecules can be labelled (in contrast to radiolabelling) in a given substance.
b. the chemiluminescent reaction velocity can be controlled (in contrast to radioactivity where one is dependent upon the half-life of the radionuclide).
c. Chemiluminescent labels are usually very stable and can be stored in solutions containing azide or merthiolate.
Disadvantages
a. The efficiency of the chemiluminescent reaction is usually very low (under 5% of the total energy released), although new compounds with efficiencies above 30% have been reported (see l.c. (16)).
b. Chemiluminescence suffers from the same disadvantages as β-scintillation counting, i.e. signal "quench" effects.

Tab. 2. Examples of some organisms exhibiting bioluminescence.

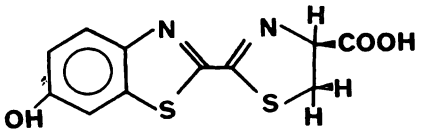
Noctiluca miliaris	– a marine dinoflagellate responsible for marine phosphorescence.
Renilla reniformis	– the sea pansy.
Diplocardia longa *Octochaetus multiporus*	– luminescent earthworms.
Latia neretoides	– a freshwater limpet.
Arachnocampa luminosa	– the New Zealand glow-worm.
Chaetopterus variopedatus	– a marine polychaete annelid.
Hoplophorus gracilorostris	– a deep-sea shrimp.
Parapriacanthus ransonneti	– a luminescent fish.
Watasenia scintillans	– a luminous squid.
Mycena citricolor	– a luminous fungus.
Photobacterium phosphoreum	– a luminescent bacterium.
Photinus pyralis	– the American firefly.

Tab. 3. Basic tenets of bioluminescent observed by *Robert Boyle* (see l.c. (1), (2)) in the 17th century.

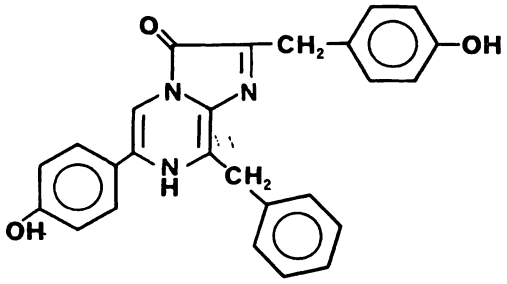
a. Light without heat.
b. Very little oxygen is needed for the maximal light output.
c. The oxygen-dependent light-reaction is reversible.
d. The light can be extinguished with chemical reagents, e.g. turpentine oil, strong spirit of salt, weak spirit of sal ammoniak.



Cypridina Sp. an ostracod crustacean

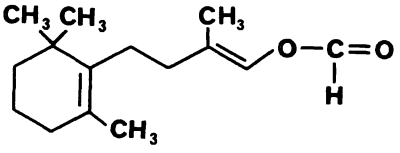


Photinus Sp. (only the *D*-form is biologically active)

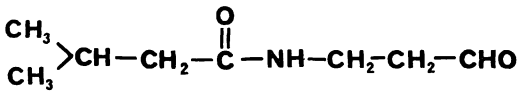


Renilla reniformis

Photobacterium Sp.



Latia Sp.



Diplocardia longa

Fig. 2. The chemical structures of several luciferins. The *Cypridina* luciferin represents the indole group, whereas the luciferin from *Photinus* is a member of the thiazole group. The *Renilla* luciferin works only in the presence of calcium ions, that from *Photobacterium* requires NAD. The *Latia* luciferin is an enol formate and *Diplocardia* luciferin requires oxygen for its activity.

Figure 3 shows the measurement of both bioluminescent and chemiluminescent reactions as practiced in this laboratory and table 4 shows the advantages and disadvantages of bioluminescent measurements.

Tab. 4. Characteristics, advantages and disadvantages of bioluminescence.

Characteristics
a. Associated with living organisms.
b. Consists of two main components, a luciferin (substrate) and a luciferase (enzyme).
c. Often, but not always, linked with ATP or NAD(P)H dependent enzymes.
d. Components usually sensitive to conventional anti-microbial agents such as azide or merthiolate.
Advantages
a. Light output highly efficient (up to 90% of theoretical values)
b. Kinetic measurements are possible.
c. Amplification effects of ATP or NAD(P)H-producing enzymes can be utilised. This can lead to more sensitive immunoassays.
Disadvantages
a. Reagents are more expensive than for chemiluminescence.
b. The enzymes are often unstable and cannot be stored in liquid state for long periods of time.
c. Preparation of "labels" usually complicated (for luminescence immunoassay purposes).

Solid Phase Luminescence Immunoassays

In contrast with other laboratories carrying out "conventional" liquid-phase luminescence immunoassays, (13, 18) the assays here described use one reaction component convalently coupled to a solid-phase, in this case, to a polystyrene ball with a diameter of 6.4 mm/0.25 inch (23, 24), (Spherotech Kugeln, Fulda, D, or Precision Plastic Ball Co. Chicago, Illinois, USA). The SPALT method published from this laboratory (14) has the antigen, or a derivative of the antigen to be measured coupled to the solid-phase whereas the ILMA and ILSA methods have the substance specific antibody (purified IgG-fraction thereof) coupled, analogous to the IRMA technique (25, 26). Other solid-phases used in luminescence immunoassays have been microcrystalline cellulose (14) and polystyrene tubes (11, 27).

The advantages of SPALT, ILMA and ILSA techniques are that they both use a "tracerless" incubation step – i.e. there is no dilution of the antigen in the sample with labelled antigen in the first incuba-

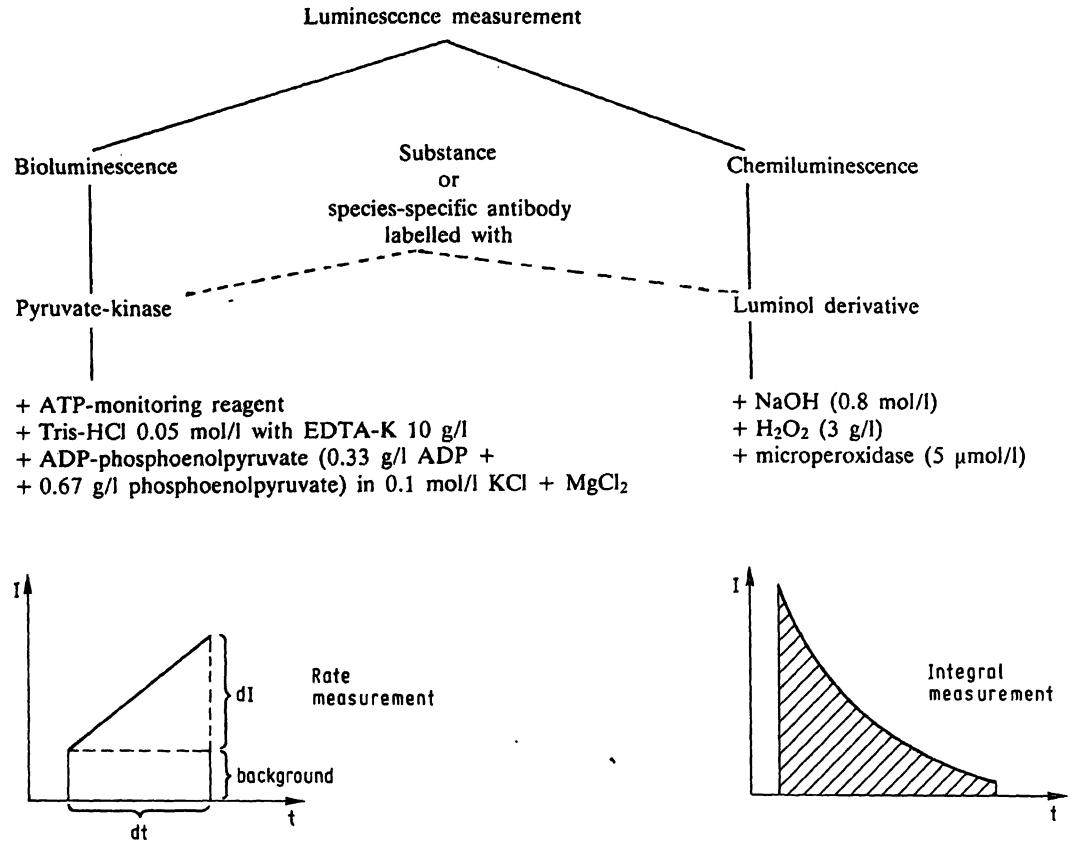


Fig. 3. The two forms of luminescent measurement used in the luminescent immunoassay sytems described in this article. The reagents, together with the concentrations used in the light-generation step have also been included.

tion step as in conventional CELIA and RIA methods. This partly explains the lower detection limits of SPALT, ILMA and ILSA for a given antigen, when compared with conventional CELIA methods. A second advantage of SPALT and ILSA is the ability to use a "universal label", in this case labelled species-IgG-specific antibody, e.g. donkey anti-rabbit IgG, which is the only tracer needed for all antibodies raised in rabbits. The label can either be chemiluminescent, e.g. diazoluminol or bioluminescent, e.g. pyruvate kinase. The disadvantage of the ILSA method is that in order to use a "universal label", the two substance-specific antibodies must come from non-related species, e.g. mouse and rabbit. Figures 4a–4d summarise the CELIA, SPALT and ILSA techniques. The choice of label for luminescence immunoassay depend to a large extent upon the chemical expertise available. The choice of diazoluminol and pyruvate kinase as labels was made because of the ease of synthesis of the antibody-luminogen derivatives (23, 28, 29). Full details of synthesis and practical application are just published (24, 25, 29).

The question bioluminescence or chemiluminescence can be answered as follows: As the bioluminescent system is more sensitive, it should be used where the chemiluminescent label is not yet sensitive enough. Bioluminescent components are more expensive and less stable in solution, than are their chemiluminescent counterparts. The cost ratio is at least 20:1 more expensive in favour of bioluminescence. The progression from bioluminescent to chemiluminescent assays can be given using this laboratory as an example. The first luminescent immunoassay for serum transferrin used a pyruvate kinase labelled antigen (28) and was used as the sole routine assay for over 12 months. As the expertise with chemiluminescent systems was improved, the assay was substituted by a bioluminescent SPALT and than a chemiluminescent SPALT, the latter being the current routine serum transferrin method, (see fig. 4c). The only bioluminescent immunoassay which has not yet been replaced by a chemiluminescent one in the author's laboratory is for serum thyrotropin levels. The reason here is that the assay,

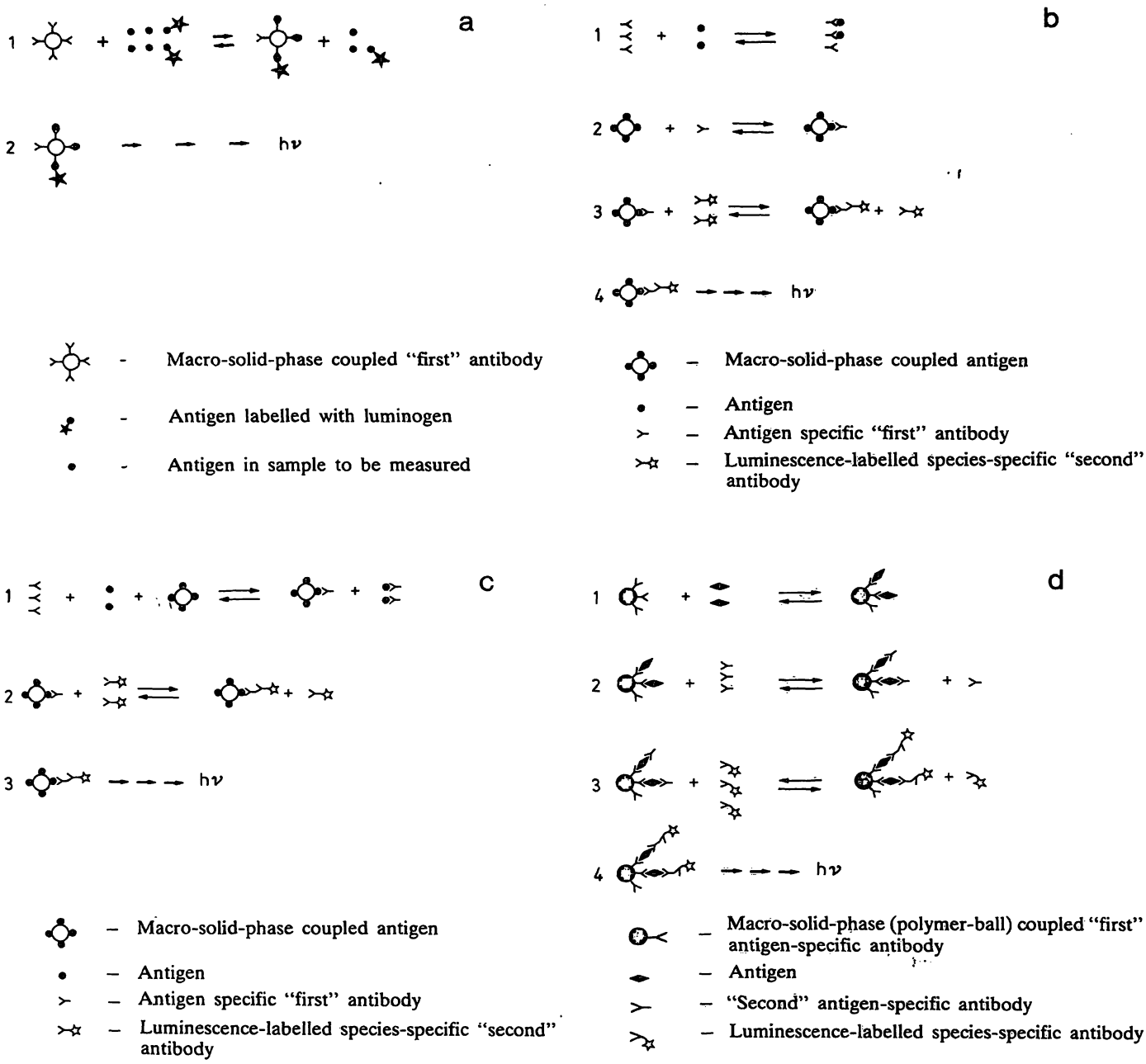


Fig. 4. Reaction schemes.
a. "Conventional" solid-phase chemiluminescence immunoassay, CELIA.
b. Sequential SPALT assay as already described (23, 26).
c. A modification of b, where the first two incubation steps have been combined. This assay can be used where the required sensitivity is easily obtained, e.g. in serum protein or drug assays.
d. ILSA principle. It is used for proteins and peptides with at least two determinants or epitopes, and where a low detection limit is needed, e.g. in proteohormone assays.
Assay a. uses a labelled antigen, assays b—d a labelled species-specific (second) antibody. Separation of unbound material is effected by washing.

which uses the ILSA technique, has two substance-specific antibodies which are not optimally matched.

To give an idea of the use of luminescent immunoassay in routine in-vitro diagnosis, table 5 gives a list of such assays either in routine use, or undergoing clinical evaluation in the author's laboratory. Table 6 shows both the advantages and disadvantages of solid-phase immunoassays.

Evaluation of Luminescent Immunoassays

The proof of the pudding is in the eating = so runs an old English proverb; and so it is with newly developed methods that they can only be accepted after they have been "standardised" against existing methods. In the case of the luminescent assay, it must be compared with RIA and other established immunological procedures. As there are only a few

Tab. 5. List of luminescence immunoassays undergoing clinical trials or already in routine use in the laboratory of the author (June 1983).

Component	Label	Assay type	Stage of development
Transferrin	Pyruvate kinase	CELIA (28)	R[+]) – disctd.[•])
Transferrin	Pyruvate kinase	SPALT	R – disctd.
Transferrin	Diazoluminol	SPALT	R – disctd.
Transferrin	Diazoluminol	SPALT (comp)[++])	R
Coeruloplasmin	Diazoluminol	SPALT (comp)	R
Orosomucoid	Diazoluminol	SPALT (comp)	CE
Transthyretin (TBPA)	Diazoluminol	SPALT (comp)	CE
Ferritin	Diazoluminol	ILSA	CE
Thyroxine binding globulin (TBG)	Diazoluminol	CELIA	CE – disctd.
Thyroxine binding globulin (TBG)	Diazoluminol	ILSA	R
Thyrotropin (TSH)	Pyruvate kinase	ILSA	D
Thyroglobulin	Diazoluminol	SPALT	CE
Thyroglobulin antibody	Diazoluminol	SPALT	CE
Insulin	Pyruvate kinase	SPALT-MCC[••])	CE – disctd.
Thyroxine (T_4)	Diazoluminol	SPALT	CE
Triiodothyronine (T_3)	Diazoluminol	CELIA	CE
Gentamicin	Diazoluminol	SPALT (23)	R
α_1-Foetoprotein	Diazoluminol	ILSA	R
Cortisol	4-Aminobutyl-N-ethyl isoluminol	CELIA	R – disctd.
Cortisol	Diazoluminol	SPALT	R

[+]) R – routine, CE – clinical evaluation, D – development stage
[•]) disctd. – assay discontinued or superseded.
[++]) competitive assay – see figure 4c
[••]) MCC – microcrystalline cellulose as solid-phase.

Tab. 6. .Advantages and disadvantages of solid-phase immunoassays.

Advantages – coated tubes

a. Ease of mechanization.
b. Reduction in the number of pipetting steps.
c. No need for centrifugation for "bound/free" separation.
d. Possibility of "sandwich-type" assays, e.g. IRMA, ILMA and ILSA.

Additional advantages – coated balls

e. Additional flexibility due to free choice of reaction/measurement vessel.
f. Possibility of "tube-change" before measurement to remove unspecific label binding to reaction vessel wall.
g. Reduction in bulk of the immobilised reactant (storage space saving!).
h. Ease of handling, and possibility of simultaneous dispensing of balls.

Disadvantages – coated tubes

a. Difficulties in mechanization of tube-activation and component coating.
b. Difficulties in obtaining acceptable precision using adsorptive techniques.
c. Limitation in the choice of measuring equipment (especially true for luminescence and enzyme immunoassays).
d. Relatively large waste of coating-solutions (high volume/surface area coated relationship).
e. Not suited for small laboratories without considerable experience in coating procedures (only for experimental and assay development purposes).

Disadvantages – coated balls

Only (b) for coated tubes applies also to coated balls.

compounds for which definitive methods are available (30), i.e. where the true value can be measured, comparisons must be made with established international reference preparations from the World Health Organisation (WHO) or the National Institute for Biological Standards and Controls (NIBSC) (formerly the Medical Research Council (MRC)) in London, GB, for peptide hormones. For comparison of hapten immunoassays, the "pure" substance can be weighed in, preferably using a human serum matrix.

As one can see, the accent must be on precision rather than accuracy. Three assays have been chosen to show the stages in assay comparison. All three have been developed by students working for their thesis in the author's laboratory.

An ILSA for α_1-foetoprotein was chosen as it was possible to compare the assay with established radio- and enzyme immunoassays as well as with the international reference preparation, WHO 72/225. An orosomucoid (α_1-acid glycoprotein) SPALT assay was chosen as a protein for which no such reference preparation was obtainable and for which the only comparison was a radial immunodiffusion method. As a hapten assay where weighed-in standards were used, as well as a commercial RIA, gentamicin was chosen as a SPALT assay.

Table 7 shows the assay schemes for α_1-foetoprotein in short and long versions, the former for pregnancy control, the latter for post-operative tumour control. Table 8 shows results from radio- enzyme- and luminescence immunoassays for selected patient samples. The correlation between all three assays was excellent when the kit standards were used for the commercial test packs and the WHO 72/225 standard for the luminescent assay. The correlation between RIA and ILSA on 40 samples for the regression y = a + bx gave the following values: r = 0.986, a = 2.80, b = 0.934. The corresponding values for EIA and ILSA were: r = 0.996, a = −1.50, b = 0.992. The radioimmunoassay kit was from Amersham (Amersham-Buchler, Brunswick, FRG) and the enzyme immunoassay kit from Abbott (Abbott GmbH, Wiesbaden-Delkenheim, FRG).

Figure 5 shows standard curves for the short and long α_1-foetoprotein ILSA using identical reagents and set up at the same time.

Table 9 shows the orosomucoid assay procedure, using commercially obtained human material (Sigma, Munich, catalogue number G-9885) for the standard curve. The range of values found in a mixed patient group including outpatients and patients in the intensive care unit was 0.24–1.90 g/l, which agrees with values to be expected ("normal range" from Behringwerke, Marburg a.d. Lahn, FRG, 0.55–1.40 g/l, mean 0.90 g/l). The number of serum samples measured was 45. The next stage is the comparison with radial immunodiffusion in a study where 50

Tab. 7. Assay scheme for the α_1-foetoprotein ILSA.

Component/Step	Assay A[+]	Assay B
Serum/sample volume (µl)	20	50
Buffer 4L + PBS/Tween (mixed in equal proportions) (µl)	300	250
Anti-α_1-foetoprotein (sheep) ball	1	1
Incubate time (h)/temp. (°C)	2/20–24	14–20/20–24
Aspirate off buffer and wash with PBS/Tween (1 ml). Aspirate and wash with 0.15 mol/l NaCl (1 ml). Aspirate.		
Anti-α_1-foetoprotein (rabbit) (µl)	300	300
Incubate time (h)/temp. °C	2/20–24	6/20–24
Wash as above		
Diazoluminol-donkey anti-rabbit-IgG (µl)	300	300
Incubate time (h)/temp. (°C)	2/20–24	2/20–24
Wash as above, transfer ball to measuring cuvette, add 250 µl 0.15 mol/l NaCl, measure in Luminometer.		

[+] Assay A was designed for pregnancy monitoring and Assay B for post-operative follow-up of hepatoma patients, or patients with pre-operatively elevated α_1-foetoprotein levels.

The sheep anti-α_1-foetoprotein was obtained from Seward Antibodies (Proma, Augsburg, FRG).

The rabbit anti-α_1-foetoprotein was purchased from DAKO (Boehringer Ingelheim, Ingelheim, FRG) and was diluted 1:500 before use.

The donkey anti-rabbit-IgG (Wellcome RD-17, Wellcome, Burgwedel, FRG) was first purified over DEAE-Cellulose, labelled with diazoluminol and passed over an Ultrogel A4 column (LKB, Munich FRG) before use. The working dilution was estimated at approximately 1:700 when compared with the starting material.

The concentration of Tween 20 in the buffers was 1 ml/l.

Tab. 8. Selected sera to demonstrate performance of the α_1-foetoprotein ILSA.

1. Test for "High dose hook" effect.
Sera from patients with hepatomas, concentration measured by dilution with normal human serum followed by radioimmunoassay (Amersham RIA-kit).

Patient 1 – concentration in serum by RIA 112.5 × 10^6 U/l
Light signal of 400 × 10^3 U/l standard in ILSA – 6200 mV · s
Light signal of undiluted patient sample – 8340 mV · s

Patient 2 – serum concentration by RIA 2560 × 10^3 U/l
Light signal of undiluted patient sample – 7355 mV · s

2. Dilution of a patient with a hepatoma to demonstrate linearity. Concentration measured by EIA (Abbott AFP-EIA kit) at a 1:10 dilution (corrected) 856 × 10^3 U/l.

Dilution factor	Concentration in ILSA 10^3 U/l	Concentration corrected for dilution, 10^3 U/l
1:1 (undiluted)	>200	–
1:2	>200	–
1:4	199	796
1:8	112	896
1:16	55.6	890
1:32	27.4	877
1:64	14.0	896
1:128	7.11	910

Samples diluted with human serum with an α_1-foetoprotein content under 10^3 U/l. Samples assayed in Assay B in table 7.

3. Serum from pregnant women (15–20th week of pregnancy).

Patient No.	RIA (10^3 U/l)	EIA (10^3 U/l)	ILSA (10^3 U/l)
1	79	71	69
2	70	77	67
3	75	69	78
4	177	192	184
5	59	55	52

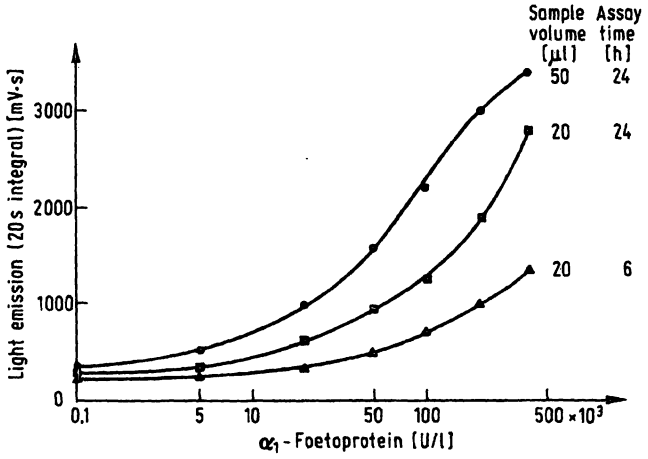


Fig. 5. The effect of serum sample volume and assay time in an ILSA for α_1-foetoprotein.

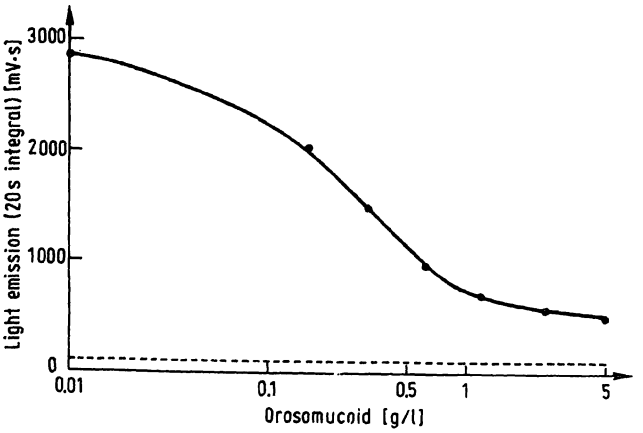


Fig. 6. Typical standard curve for a SPALT assay for orosomucoid (α_1-acid glycoprotein) in which standards and sample are pre-diluted 1:25 before assay. The dotted line shows the unspecific binding, i.e. where the substance-specific antibody has been omitted from the system. It represents the adsorption of label to the balls and is under 0.1% in an optimised system.

routine sera are exchanged, each party only knowing his obtained values. This somewhat shaky comparison must unfortunately suffice for the moment, although it is far from an acceptable solution. Figure 6 shows an orosomucoid standard curve.

Table 10 shows the optimised gentamicin SPALT which has replaced its forerunners (14, 23). Figure 7 shows a standard curve and table 11 quality control data. This assay is in routine use and has fulfilled the requirements of an independent external quality control programme, (INSTAND, Düsseldorf, FRG).

The gentamicin assay has been chosen to show the precision and accuracy (against weighed-in standards) of solid-phase luminescence immunoassays using the equipment at present available for measurement. All data used here was obtained from an LKB-1251 luminometer.

Tab. 9. Assay scheme for the orosomucoid (α_1-acid glycoprotein) SPALT.

Component/Step	Volume/Time
Sample/Standard (prediluted 1:25 with buffer 4N) (µl)	50
Rabbit anti-human orosomucoid (1:500 in buffer 4N) (µl)	250
Incubate at ambient temperature (min)	10
Add 1 orosomucoid-coated polystyrene ball and gently agitate.	
Incubate at ambient temperature (min)	120
Wash as in the α_1-foetoprotein assay (see tab. 7)	
Donkey anti-rabbit-IgG-diazoluminol (µl)	300
Incubate at ambient temperature (min)	120
Wash as above, transfer ball to clean measuring cuvette and insert in luminometer after adding 250 µl 0.9 mol/l NaCl to keep ball wet.	

The donkey anti-rabbit-IgG-diazoluminol was identical with that used in the α_1-foetoprotein assay described in tab. 7.
The addition of the NaCl to keep the ball wet improved assay precision.

Tab. 10. Assay scheme for the Gentamicin SPALT in routine use.

Component/Step	Volume/Time
Sample/Standard (1:450 dilution in 0.15 mol/l NaCl) (µl)	50
Buffer 4L + PBS-Tween (see tab. 7) (µl)	200
Anti-gentamicin (rabbit) 1:125 dilution in buffer 4N (µl)	50
Incubate at ambient temperature for 15 min	
Add a gentamicin-coated polystyrene ball	
Incubate at ambient temperature for 35 min	
Wash as in α_1-foetoprotein ILSA (see tab. 7)	
Donkey anti-rabbit-IgG-diazoluminol (µl)	300
Incubate at ambient temperature for 60 min	
Wash as above, transfer ball to cuvette, add 250 µl 0.15 mol/l NaCl and measure in luminometer.	

The donkey anti-rabbit-IgG-diazoluminol used was identical to that in the α_1-foetoprotein assay in table 7.
The "gentamicin-coated ball" was coated with a gentamicin-bovine serum albumin complex to complement the gentamicin-transferrin conjugate used for immunising the rabbits.

Tab. 11. Selected quality-control parameters for the gentamicin SPALT.

1. Standard curve data

a. Intercept stability, data from 20 assays

80% intercept – 0.893 µg/l ± 0.103 µg/l (CV 11.5%)
65% intercept – 1.58 µg/l ± 0.130 µg/l (CV 8.19%)
50% intercept – 2.62 µg/l ± 0.137 µg/l (CV 5.24%)
35% intercept – 5.29 µg/l ± 0.389 µg/l (CV 7.35%)
20% intercept – outside the standard curve (above 16 µg/l)

b. Unspecific binding data from 20 assays – ratio to B_0 and B_{16}.

Ratio B_0/UB – 22.6 ± 2.07 (CV 9.16%)
Ratio B_{16}/UB – 5.43 ± 0.716 (CV 13.2%)

These figures are related to the sensitivity of the luminometer and represent the signal to noise ratios at the zero and 16 µg/l standard curve points (see tab. 12 for corresponding data for the LB 950).

2. Intra- and inter-assay coefficients of variation for three control sera. Data from 20 samples (intra-) from mean of duplicates (inter-assay).

Intra-assay	Serum IV	Serum V	Serum VI
Mean (µg/l)	2.21	7.04	0.55
Standard deviation (µg/l)	0.042	0.371	0.027
Coefficient of variation (%)	1.93	5.27	4.91
Inter-assay			
Mean (µg/l)	2.26	7.14	0.59
Standard deviation (µg/l)	0.078	0.659	0.045
Coefficient of variation (%)	3.46	9.23	7.62

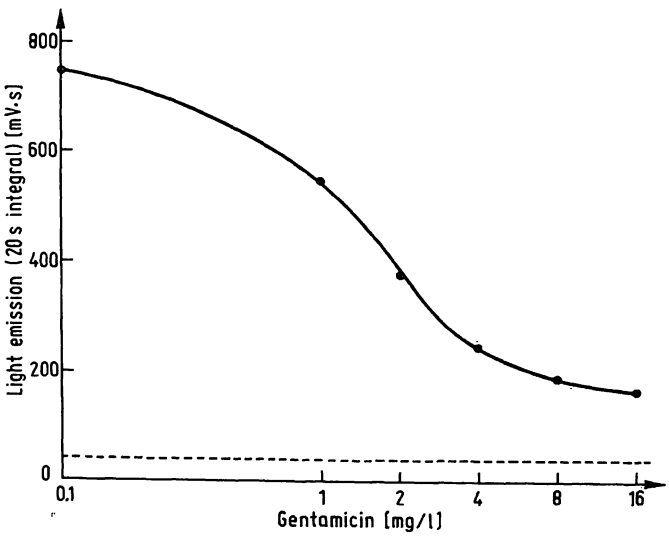


Fig. 7. Gentamicin SPALT assay which has been designed to measure "trough" values (values directly prior to drug administration). The area of interest covers the range up to 4 mg/l, trough values above 2 mg/l being regarded as too high.

Problems with Luminescence Immunoassays

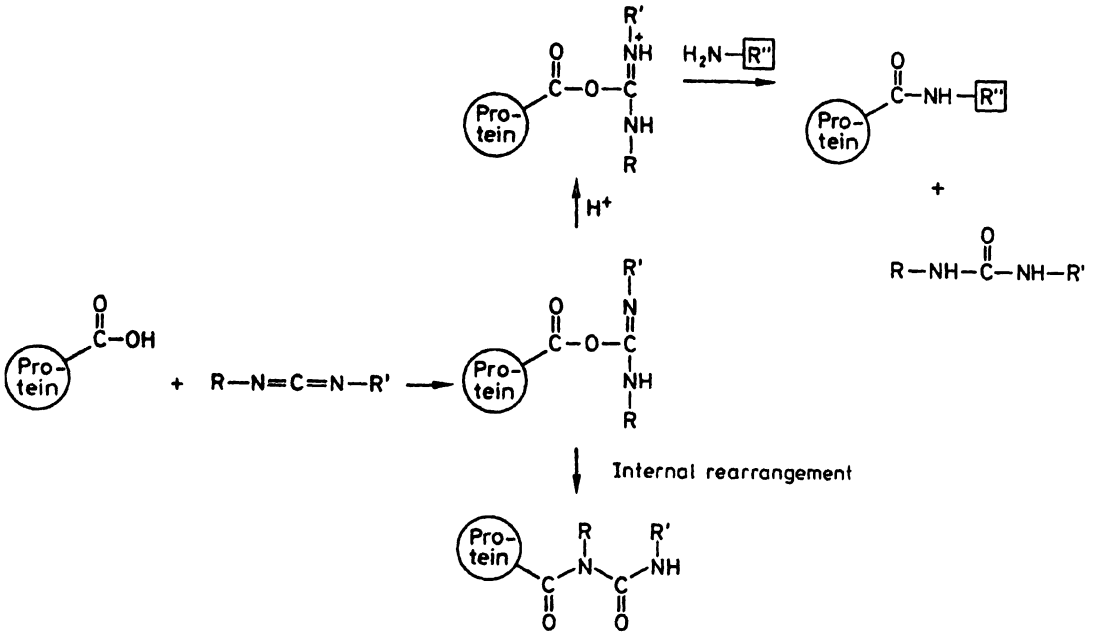
The problem with bioluminescent labels have been dealt with inasmuch as they are relatively unstable and cannot be conserved with the usual anti-microbial agents such as azide or merthiolate. Chemiluminescent compounds, when used in liquid-phase immunoassays suffer from the same drawbacks as β-emitting radioisotopes such as ^3H or ^{14}C, namely quench effects due to serum components which absorb light of the same wavelengths as that emitted by the luminescent compound. These effects can be minimised by using solid-phase immunoassays (26, 27) or by extracting the components to be measured prior to assay (27).

The main problems encountered in assay development in this laboratory have been of a chemical nature, especially when working with hapten SPALT assays. Here the structure of the immunogen is of utmost importance as is the structure of the hapten-protein complex coupled to the solid-phase, especially where carbodiimides are used as coupling reagents. The effect of the "bridge" between hapten and protein in tracer-binding in RIA is well known (31), and figures 8 and 9 show the reactions occurring with carbodiimides and the effects of immunogen and immobilised hapten-protein on the standard curve produced (26).

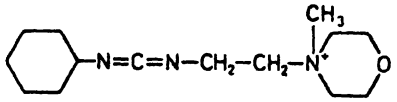
Practical problems which can arise in luminescence immunoassays can be demonstrated using the gentimicin SPALT described earlier. The precision of the assay was not only influenced by the incubation times and wash solutions used, but also by the state of the solid-phase before measurement of the luminescent signal. The coefficient of variation obtained using dry balls was more than double that obtained when the bells were kept covered with physiological saline (dry balls coefficient of variation (CV) 8.7%, moist balls CV = 6.9%, balls in saline CV = 4.0% – all values being those at a concentration of 6.0 mg/l gentamicin).

Problems in using coated tubes, especially for steroid assays, have already been published (32) with regard to unspecific binding of tracer and with regard to the efficiency of adsorption of antibody depending upon the ionic strength and pH of the coating solution (33).

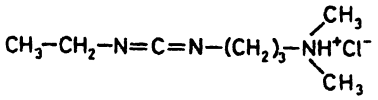
When using the ILSA technique, the substance specific antibodies must come from two unrelated species in order to be able to use a labelled antibody directed against the "outwards-pointing" antibody. An alternative to this is to use a modified ILMA in which the "outwards-pointing" antibody has been labelled with N-hydroxysuccinimidyl-biotin. In this



Water-soluble carbodiimides



1-Cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

Fig. 8. Reaction scheme showing the two possibilities which can occur when using carbodiimides for coupling haptens to proteins. In one case (shown here as internal rearrangement) the carbodiimide becomes attached to the protein and can act as a potent antigen. In such cases, antibodies are raised to the hapten as well as to the rearranged carbodiimide.

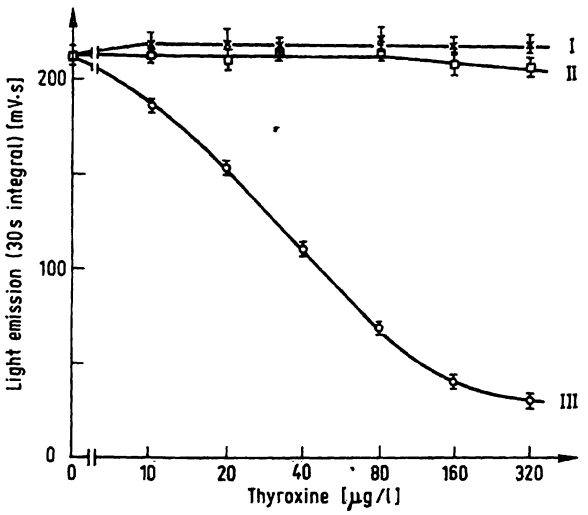


Fig. 9. The importance in the choice of immunogen and protein-hapten conjugate for coating balls for a hapten-SPALT in practical terms. In case I, identical conjugates were used as immunogen and for ball-coating. In case II a transferrin T_4 conjugate was used to coat the balls, the conjugate being formed with the same carbodiimide as the immunogen. In case III the hapten-protein conjugate for ball-coating was identical to case II with the exception that another carbodiimide had been used for coupling. Figure 9 shows the practical consequences of figure 8! Measured with LKB 1250.

case, the species-specific antibody label is replaced by diazoavidin, avidin N-(4-aminobutyl-N-ethyl)isoluminol hemisuccinamide or avidin pyruvate kinase. A biotin-avidin coupled system can be used in CELIA systems where the ligand to be measured (e.g. insulin) can be biotin labelled. The amplification effect necessary is obtained from the avidin-luminogen complex. The latter has the advantage that the serum sample and luminogen can be kept separate, thus reducing potential interference from serum components.

Another labelling possibility is to label peptides directly with N-(4-aminobutyl-N-ethyl)isoluminol hemisuccinamide or diazoluminol, although this is of minor interest when SPALT or ILMA assays are possible. Newer results have shown that all ILSA assays using diazoluminol as label can be replaced with ILMA assays in which the antibody is labelled with N-(4-aminobutyl-N-ethyl)isoluminol hemisuccinamide (26, 29). The advantage gained here is that the antibodies at both sides of the sandwich can come from the same species, as is the case in the thy-

roglobulin ILMA. The use of N-(4-aminobutyl-N-ethyl)isoluminol hemisuccinamide via an active ester (N-(4-aminobutyl-N-ethyl)isoluminol hemisuccinamide N-hydroxysuccinimide ester) allows a high incorporation of luminogenic groups without loss of solubility and immunological activity. This results in assays with lower detection limits than for assays using diazoluminol as label.

Instrumentation

The instrumentation for luminometry is still in the early stages of development, especially where immunoassays are concerned. At present only semi-mechanized luminometers are available, mainly with a limited sample capacity (under 50 samples) which precludes their use for long routine series.

The author's laboratory has experience with two semi-mechanized luminometers, the main features of which are shown in table 12. Neither machine is able to process the raw data fully so that a manual entry of "counts" must be made into a desk-top computer with modified RIA-data processing programme. One cannot expect a laboratory with automatic RIA equipment to change over to a semi-mechanized luminometer, even when the assay procedure is as easy or easier than the original RIA. In the case of the LB 950, extensive experimenting and modification was needed until the accepted reliability and precision was attained so that routine assays were possible without the assistant having to stand over the machine to make sure that nothing went wrong!

The LKB-1251 is built to such fine tolerances that small deviations in the cuvette diameter lead to

Tab. 12. Major features of the LKB 1251 and LB 950 luminometers.

	LKB 1251	LB 950
1. General		
Capacity – no. of samples	25	300
Cuvette size (mm)	51 × 12	37 × 12 – 55 × 12
Special cuvettes	Yes	No
No. of injectors (maximum)	3	3
Pump-type	Peristaltic	Syringe
Pump-speed variable	No	Yes
Pump-volume variable	Yes	Yes
Mixing possible during injection/measurement	Yes	No[+])
Pump volume maximum (µl)	ca 50	350 (700[++]))
Repeat-dispensing possible	Yes	No
2. Measurement and Electronics		
Dialogue with luminometer possible directly	Yes	Only via computer
Software for automatic data processing for luminescence immunoassays included	No	No
Reaction kinetics displayable	via plotter	on VDU
Data/Programme storage	EEPROM	Diskette
Computer/Microprocessor delivered with luminometer	Built-in Microprocessor and RS 232-C outlet	Apple II or Basis 108
Selected intention times possible	Yes	Yes
Kinetic (rate) measurements possible	Yes	Yes
3. Performance (in terms of gentamicin SPALT run with identical reagents at the same time by the same technician		
CV of reagent blank (enzyme oxidant blank) (%)	1.39	2.04
CV of sample with 2.2 µg/l (n = 20) (intra-assay variation) (%)	1.93	2.39
Ratio of signals (see also tab. 11)		
B_0/UB	22.6	9.49
B_{16}/UB	5.43	2.87
B_0/EOB	62.4	62.6
UB/EOB	2.93	6.59

[+]) There is the possibility of blowing compressed air into the measurement chamber to effect mixing of the reagents.
[++]) The LB 950 in use has been modified as follows: All syringes have been replaced by syringes with Teflon plungers (Hamilton, Bonaduz, CH), the volume of the syringe for injecting into the measurement chamber being increased from 0.5 ml to 1.0 ml.

blockage of the transport system. This means that only high-quality cuvettes (LKB or Sarstedt No. 68.750) can be used. The advantage of the LB 950 is that high quality test tubes of diameters 11–12 mm and length 37–55 mm can be used. Here again, the emphasis is on the quality, as many test tubes tried had unacceptable tolerances so that only tubes from Sarstedt (55 × 12 mm No. 55.484) were used.

Over 4000 samples were run in parallel on both LKB-1251 and LB 950 to test for precision and dynamic range. After optimisation of both luminometers, no difference in performance could be seen. The LKB 1251 was used for short experimental series, the LB 950 for longer routine luminescence immunoassays. In order to make luminescence immunoassays an interesting alternative to RIA the instrumentation may have to be presented in the form of "Black Boxes" with high reliability and throughout as the main criteria.

Commercial Kits

Although there are no commercial luminescence immunoassay kits on the market at the time of writing, it is to be expected that they will soon make their debut. Important points to be considered are, for example, if coated tube assays are to be produced, will the tubes fit into all luminometers? The answer is at present definitely no, and it is easy to see, that if coated ball assays were developed, that this problem is automatically bypassed. Similarly, liquid phase assays of the CELIA-type also circumvent the need for special cuvettes.

Commercial kit producers do not seem to have realised the full potential of luminescence immunoassays, and are no doubt waiting for the first firm to launch itself into this field, where the "patent-density" is still relatively thin!

Conclusions

This article has been intended go give a brief overview as to what is happening in the field of luminescence immunoassay, and is intended to stimulate the reader into action in this field! Although is is now evident that luminescence immunoassays can replace other immunoassays in all sectors (26) the impact of such assays will depend on their commercialisation. Parallel to the development of robust kits, the luminometer producer must present an instrument which has all the comforts of the present-day RIA-and EIA-automatic assay and data-reduction systems.

Acknowledgements

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Marker-Free Immunological Analytical Methods[1])

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Summary: The antigen-antibody reaction itself acts as the "marker system" in the marker-free immunological methods. This group of analytical techniques includes radial immunodiffusion, nephelometry, turbidimetry and nephelometric inhibition. The Particle Counting-Immunoassay (PACIA) technique also belongs to this group, although it employs latex particles as markers for the intensification of the indicator reaction.

The principles of the above methods are described, and their applications in diagnosis, their detection limits and important interfering factors are discussed. For all methods, the precision in series and from day to day is in the range of 5–10%. The detection limits are 1 ng/l for the PACIA technique, 10 µg/l for nephelometric inhibition, 1 mg/l for nephelometry as an end point technique, and 10 mg/l for nephelometry as a kinetic technique.

Commercial sources of reagents and apparatus are given in an appendix.

Markerfreie immunologische Analysenverfahren

Zusammenfassung: Die Antigen-Antikörperreaktion stellt für die markerfreien immunologischen Analysenverfahren das „Markersystem" dar. Zu dieser Gruppe von Analysenverfahren werden die radiale Immunodiffusion, die Nephelometrie, die Turbidimetrie, die nephelometrische Inhibierungstechnik und die Particle Counting Immunoassay (PACIA)-Technik gezählt. Letztere verwendet allerdings zur Verstärkung der Indikatorreaktion Latexpartikel als Marker.

Die Prinzipien der genannten Verfahren werden dargestellt, die diagnostische Bedeutung, Nachweisgrenzen sowie die wesentlichen Störfaktoren werden diskutiert. Die Präzision in der Serie und von Tag zu Tag liegt für alle Verfahren im Bereich von 5 bis 10%. Die Nachweisgrenzen für die PACIA-Technik liegt bei 1 ng/l, für die nephelometrische Inhibierung bei 10 µg/l, für die Nephelometrie als Endpunkttechnik bei 1 mg/l und für das kinetische Verfahren der Nephelometrie bei 10 mg/l.

In einem Anhang werden die Bezugsquellen für Reagenzien und Geräte aufgeführt.

Introduction

Marker-free immunological methods use the primary reaction between antigen (Ag) and antibody (Ab) as an indicator reaction. The following reaction scheme forms the basis of the method:

$$Ab + Ag \rightarrow AbAg$$

$$nAbAg \rightarrow (AbAg)n$$

$$x(AbAg)n \rightarrow \text{precipitate}$$

In the present contribution only those methods are presented that are normally found in the routine laboratory.

In radial immunodiffusion, electroimmunodiffusion and counter current electrophoresis, precipitate formation (the 3rd step of the above reaction scheme) is used for the detection of the reaction. For optimal precipitate formation, all reactants must be present in mutually compatible concentrations. this accordingly places a restriction on the lower detection limit for these analyses.

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

Radial Immunodiffusion

In this procedure, the formation of precipitation lines in a suitable medium of agar or agarose serves as the marker. Precipitate formation depends on the following factors:

1. molecular weight,
2. protein concentration,
3. agar concentration in the gel,
4. ionic strength,
5. incubation temperature.

Anitibody is present in the agar in a defined concentration. Antigen diffuses radially from a punched circular well into the agar medium. A stable ratio of antigen and antibody is attained, and a ring shaped region of precipitation becomes visible. At the end of the reaction, the logarithm of antigen concentration is proportional to the diameter of the precipitation ring. The diameters of the sample and standard precipitate rings are measured, and the antigen concentrations of the samples are determined from a standard curve.

Two evaluation procedures are commonly used:

1. the technique of *Mancini* (1), based on measurements after diffusion is complete, and
2. the kinetic procedure of *Fahey & McKelvey* (2).

Different diffusion times are observed for the various plasma proteins, e.g. 24—50 hours for IgG, and 50—80 hours for IgM and α_2-macroglobulin. These differences in diffusion time depend essentially on molecular size or molecular weight. Under certain conditions, for example in the presence of 7S IgM which has a lower molecular weight than normal IgM, the accelerated diffusion gives the false impression of increased protein concentration. Conversely, the presence of high molecular weight secretory IgA or immune complexes results in the recording of erroneously low antigen concentrations. The lower detection limit of radial immunodiffusion is about 20 mg/l; the procedure is capable of an in series precision of 2—5%, while precision day to day is in the order of 3—7% (3).

In the *Fahey* method, the diffusion time is short, so that the strict control of reaction time and temperature, and the exact measurement of the sample are very important. This procedure has the same detection sensitivity as the *Mancini* technique. Precision from day to day is in the range of 3—7%. In practice, both methods have advantages and disadvantages. The presence of rather high antigen concentrations may lead to premature reading of the results, with consequent inaccuracies.

For the determination of especially low concentration ranges in cerebrospinal fluid, urine, venous umbilical blood and venous punctates, so-called LC-plates (low-concentration-plates) are used.

The quality of the standard is critically important for the quantitative evaluation, and it should be based on the recommendations of the WHO. To obtain the highest possible precision, the diameter of the precipitation ring should be determined with the aid of a measuring microscope, or at least with a measuring lens, to an accuracy of ± 0.1 mm.

Since the plates must be evaluated visually, the quality of the results can also be judged by inspection, and any possible sources of error will be apparent.

Asymmetric precipitate rings can be caused by the following faults:

1. protuberances or indentations in the agar,
2. inadvertant application of sample outside the starting well,
3. damage by freezing of the plates,
4. separation of the agar layer from the base, and diffusion of the sample underneath the agar.

Elliptical rings are caused by:

1. overfilling of the starting well,
2. non-horizontal positioning of plates during the diffusion process,
3. partial drying out of the plates, e.g. by exposure to the sun.

Weak precipitate rings are found under the following circumstances:

1. antigen excess (M-protein),
2. antibodies of the patient against other proteins of the antiserum.

Double or triple precipitation rings are caused by:

1. multiple filling of the starting well,
2. proteolytic cleavage products of proteins,
3. unspecific (inappropriate) antisera,
4. cryoglobulin precipitation,
5. protein precipitation at the start
6. reaction with rheumatoid factors (IgM antibodies),
7. presence of circulating immune complexes.

Table 1 lists the analyses that are at present performed by immunological methods, based on RID and nephelometry, and to some extent turbidimetry. The appendix lists the commercial sources of reagents and apparatus.

Tab. 1. List of proteins that can at present be determined by RID and nephelometry.

Albumin	Gc-Globulin
Antithrombin III	Haemopexin
α_1-Antitrypsin	Haptoglobin
Apolipoprotein A-I	Human-placental lactogen
Apolipoprotein B	IgA
C_1-Inactivator	IgD
C3c	IgE
C4	IgG
(Factor VIII) (associated protein)	IgM
Coeruloplasmin	Lactoferrin
C3-Activator	β-Lipoprotein
C-reactive protein (CRP)	Lysozyme
α-Foetoprotein	α-Macroglobulin
Fibronectin	Plasminogen
Fibrinogen	Prealbumin
Acidic-α_1-glycoprotein	Prothrombin
α_2-HS-glycoprotein	Retinol binding protein (RBP)
β_2-Glycoprotein I	Thyroxin binding globulin (TBG)
β-SP-1-glycoprotein	Transferrin
α-PA-glycoprotein	

Application

Radial immunodiffusion is used for the determination of specific proteins from various sources. More recent diagnostic procedures, e.g. the determination of apolipoproteins, are also possible with the aid of this technique. The method is simple to apply and requires little space. As mentioned above, the plates also provide more information about the quality of the results than can be obtained with other methods. Several operational procedures are involved in one analysis, which means that the method is costly in operational time. A further disadvantage is that the method does not lend itself to mechanization.

Developmental trends

As an alternative to RID, nephelometry requires an investment in apparatus of between 30000 and 90000 DM. Partly mechanized clinical chemical apparatus, which operates on an exact time cycle, enables the turbidimetric determination of the most important specific proteins, without additional investment. Within a reasonable space of time, it will therefore be possible to perform all immunological analytical methods by RID or nephelometry. The range of reagents for these procedures will also be extended by the introduction of new tests.

In the routine clinical chemical laboratory, electroimmunodiffusion (rocket electrophoresis) (4) does not play a major role. The preparation of the plates is work intensive, and high quality results are difficult to obtain. This very demanding procedure is firmly established in laboratories of scientific protein research, where multidimensional techniques are favoured for the characterization of proteins and for testing the homogeneity of protein preparations.

Nephelometry

Principle

In a "clear solution", particles of a certain size cause a scattering of incident light. Usually, the wavelength of the scattered and incident light is the same. To a small extent, light of longer wavelength, i.e. of lower energy, is also emitted (*Raman* effect).

Condensed monochromatic light of a certain wavelength causes polarization of the molecules of particles in a solution. Oscillations then result in the emission of light of the same wavelength as the excitation light.

If the particle size is in the order of 0.1 of the incident wavelength, all particles are excited in the same way, and *Rayleigh* scattering (5) occurs.

Equation

$$i_\Theta = I_o \frac{8\pi^2 \alpha^2}{r^2 \lambda^4} (1 + \cos^2 \Theta)$$

Providing the excitation is performed with non-polarized light, the intensity of scattered light is proportional ($1/\lambda^4$) to the incident wavelength. The intensity increases with decreasing wavelength. The scattering diagram (fig. 1) shows that the distribution of scattered light is symmetrical, and the same quantities of light are scattered forwards and backwards.

With increasing particle size, the nature of the light scattering changes with respect to the wavelength of the incident light. The symmetry around the 90 degree axis is retained, but the ratio of backwards to forwards scattering is changed. For this reason the photomultiplier is placed at an angle of 15 to 30 degrees to the axis of the incident light. Figure 2 shows the type of light scattering that exists in the nephelometric measurements in immunological tests.

If the particles are larger than the wavelength of the incident light, as in the determination of suspended bacteria or blood cells, then *Mie* scattering (6) occurs. The diagram for *Mie* scattering is shown in figure 3.

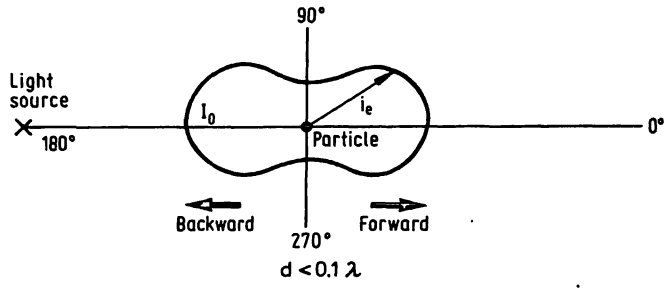


Fig. 1. Light scattering by a particle (P) of size less than 0.1 λ.

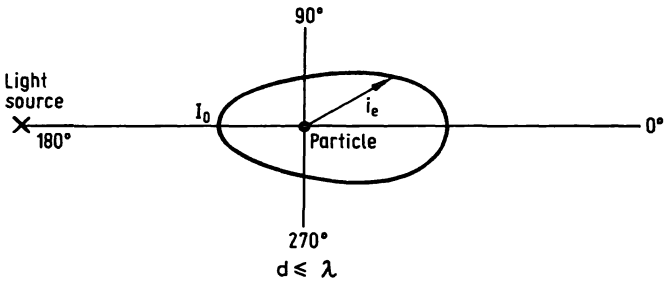


Fig. 2. Light scattering by a particle when the particle size is the same as, or only slightly less than λ.

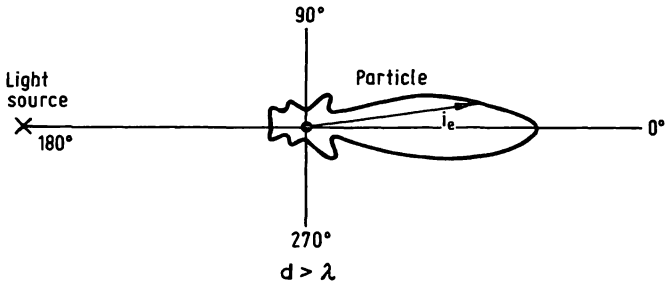


Fig. 3. Light scattering by a particle (P) when the particle size is greater than the incident wavelength.

Light scattering by antigen-antibody complexes

The antigen-antibody reaction proceeds in several stages. The rate of formation of antigen-antibody complexes depends on temperature, pH, ionic strength and the relative quantities of reactants. Antigen-antibody complexes are always lager than 0.1 λ.

Addition of 4% polyethyleneglycol (PEG 6000) accelerates and intensifies the reaction (7).

Apparatus

Nephelometric determination of the complexes may be performed kinetically (8), or by the end point method after incubation for 30 to 60 minutes (9); much less sample is required for the kinetic method. The layout of a nephelometer is shown schematically in figure 4.

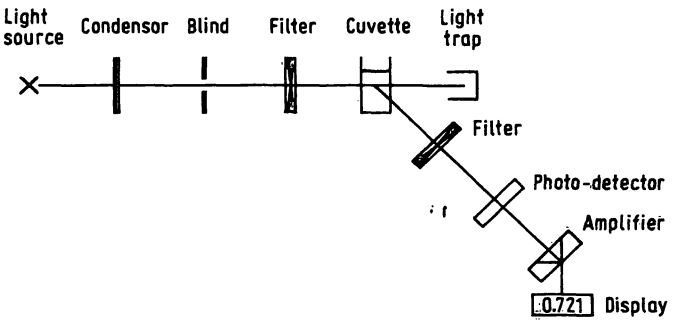


Fig. 4. Schematic representation of a nephelometer.

The light source is either a halogen lamp or an helium-neon laser. Practically all nephelometers can be converted to partly or fully mechanized systems, which perform all steps from sampling, reagent selection and dispensation to the calculation of the results.

Reagent packs are available for all specific proteins, and the range is continually being extended.

For all apparatuses and procedures, the precision in series shows a standard deviation of 3—5%, while day to day precision is 4—7% (3). Nephelometry involves the same problems of standardization as already encountered in radial immunodiffusion. The accuracy of the nephelometric determination is similar to that of RID.

The lower detection limits are 10 mg/l for the kinetic method and 1 mg/l for the end point method.

Nephelometry has the following advantages over radial immunodiffusion:

1. rapid availability of results,
2. simple mechanization,
3. short incubation times.

These advantages must be set against certain notable disadvantages. In the first place, capital investment costs are in the order of 30000 to 90000 DM, which can be justified only for large analytical loads and a demand for the rapid production of analytical results.

In the presence of an excess of antigen, the immune complex redissolves and erroneously low results are obtained. Excess antigen is tested for by a further reaction step, involving the addition of extra antibody.

Each commercially available apparatus has its own specially designed reagents, so that it is very inconvenient to adapt to a new test, and for reasons of cost or comparability of analytical results, it is difficult to use the tests supplied by other manufacturers, or to prepare reagents in the laboratory.

Since RID naturally involves visual inspection of the plate, it permits the detection of atypical precipitate rings and other errors. Nephelometry, on the other hand, provides no control over the presence of unspecific reactions.

Turbidimetry

In contrast to nephelometry, turbidimetric methods measure the decrease in intensity of incident light. Accordingly, a turbidimeter has a similar construction to that of a photometer. By the preparation of highly pure antisera, and especially by the production of clinical chemical equipment with precise timing facilities, turbidimetric procedures have found a very wide application.

The decrease of light intensity corresponds to an apparent absorbance

$$I = I_o \cdot e^{-TS},$$

where I_o is the intensity of incident light, I corresponds to the transmitted light, and S is the light path of the cuvette. T is the turbidity coefficient, and it depends on the wavelength, the concentration and the type of particles.

Test packs are available for the most important specific proteins that are present in sufficiently high concentrations for turbidimetric assay. If the tests are performed with mechanized apparatus, similar precision and accuracy are obtained with nephelometry and RID. Turbidimetry has a shorter linear measurement range than nephelometry. Nephelometry and turbidimetry require high affinity antisera, which are prepared by adsorption, affinity chromatography and ultrafiltration. All reagents of nephelometric quality are also suitable for turbidimetry.

Turbidimetry also has the same limitations as nephelometry with respect to the occurrence of undetected interference. Attention should also be drawn to the unspecific precipitation of proteins (e.g. fibrinogen) by PG 6000, which is added to accelerate the reaction; or the fluorescence of pharmaceuticals, which can be caused especially by the UV light favoured for turbidimetric measurements.

Turbidimetric analyses can be performed with "fast analysers" and automated analysers, e.g. the ACP 5040.

Nephelometry and turbidimetry require about 5 times more antiserum than does radial immunodiffusion.

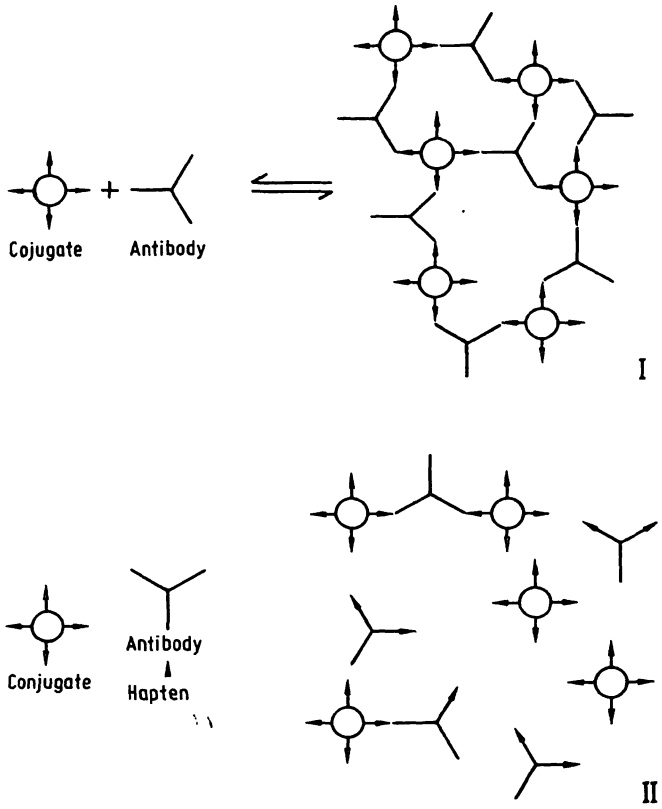
Nephelometric Inhibition Method

The methods described so far are used for the quantitative determination of macromolecular substances on the basis of their antigenic properties. Low molecular weight compounds, e.g. pharmaceuticals, have not antigenic properties. These haptens become antigenic when they are coupled to bovine albumin, and they can be used in this way for the preparation of antibodies. Thus, the immunological determination of pharmaceuticals is possible, and it is performed chiefly by radioimmunological or enzymeimmunological methods. If relatively high concentrations of pharmaceuticals are present in blood, they can be determined by the nephelometric inhibition technique. The nephelometric inhibition immunoassay was developed at about the same time by two independent research groups (10, 11).

Principle

Hapten and corresponding antibody are present in the reaction mixture. Depending on the antigen concentration, antigen-antibody complexes are formed, but no signal is produced, because the antigen is of low molecular weight. A response is not obtained until a complex of the hapten with another macromolecule (e.g. ferritin) is added. The added macromolecule-linked antigen forms a nephelometrically detectable complex with the remaining free antibody. The quantity of free antigen in the initial reaction mixture determines the quantity of macromolecule-linked antigen (starter antigen) that can become bound and thereby give rise to a signal. Figure 5 shows the reaction principle and a calibration curve.

By using this technique the sensitivity is increased by a factor of 1000; the detection limit is 10 µg/l. An in series precision of 5% is possible, and day to day precision is 8–10%. For technical reasons, the sensitivity is not in the same order as that of the radioimmunological test. Variable protein binding by some pharmaceuticals is a source of error; the resulting complexes may act as starter antigens and contribute to signal generation, thereby leading to falsely high results. This source of error can be excluded by prior extraction of pharmaceuticals from the sample; in addition, the resulting enrichment of the analyte leads to an increase in the sensitivity of the assay.



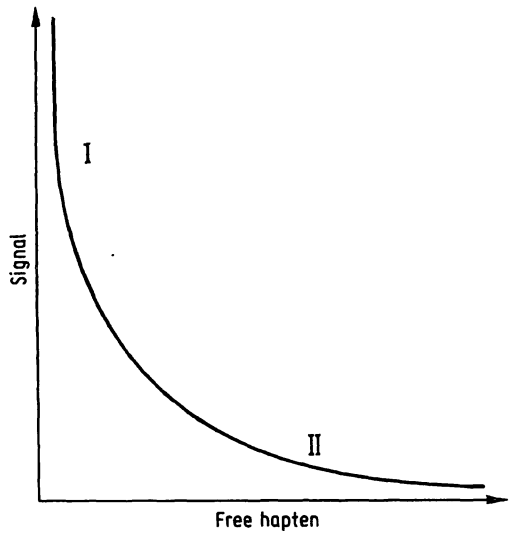


Fig. 5. Principle of the nephelometric inhibition technique.

assay mixture causes the agglutination of several latex particles. After the reaction, a wide range of particle sizes is present. Quantitation is performed with a particle counter with dark field illumination. Unspecific reactions are suppressed by the reaction conditions (pH, ionic strength and surface treatment of the latex particles). Macromolecular reactants become bound directly to the surface-treated latex particles. Haptens are linked to the latex particles with the aid of ferritin molecules. In both cases, the immunological reactivity is not affected by linkage to the latex. The method has the same sensitivity range as radio- or enzymeimmunological procedures.

The latex aggregates or reaction products are determined in a Technicon continuous flow system, linked to a dark field illuminated cell counter. The threshold is set at about 1.2 µm. After the reaction is complete, the signal decreases, because the number of free particles decreases. Figure 6 shows the principle of the PACIA technique. Compared with the capacitive technique, the optical measurement has the

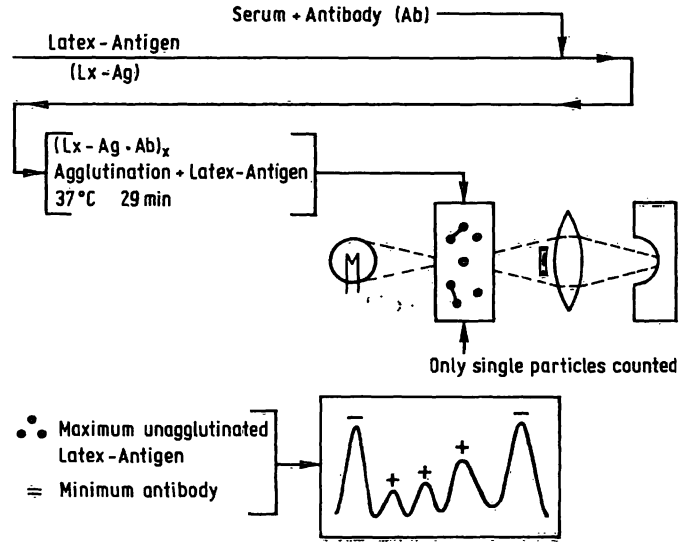


Fig. 6. Schematic representation of the PACIA apparatus from TECHNICON from (13).

Nephelometric Determination of Antigens with Latex Particles (PACIA Method)

Principle

The PACIA method (particle counting immunoassay) was developed (12) to increase the sensitivity of the antigen-antibody reaction. It is suitable for the determination of both antibodies and antigens, and is based on a similar principle to the latex test. Carboxylated latex particles are loaded with antigen or antibody. The corresponding reaction partner in the advantage of interference-free counting. Automation of the procedure guarantees the most reliable results.

The detection limit is 1 ng/l. Precision in series and from day to day for alpha-foetoprotein are 2—12.9% and 5.8—12.8%, respectively. If very low concentrations are present, the possibility of a prozone effect must be borne in mind. Other interfering factors, e.g. agglutination of the latex particles, reaction with the complement system of IgM rheumatoid factors are largely avoided by coupling the IgG-F

(ab')2-fragment to the latex particles and by changing the ionic strength.

This method promises well for future developments. A number of tests have so far been introduced, ranging from kits for the determination of circulating immune complexes to methods for the determination of insulin or pharmaceuticals, e.g. digoxin.

The equipment first developed by Technicon was discontinued, and it has only recently become available again from the firm of Acade in Brussels, under the name of IMPACT. Technicon now uses the PACIA reagents on the Random Access Analyser RA 1000 with the customary photometric technique. In the same way, it may be possible to use this sensitive method with other conventional analytical instruments. It is to be expected that this new method will be subject to far fewer problems than the other immunological analytical methods, whose inadequacies with respect to reagent storage and availability, and mechanization of analysis are well known.

Future Perspectives

At present, acceptable solutions exist to all problems concerning the available tests, the detection principle and the mechanization of the individual analytical steps. For certain levels of analytical throughput, however, mechanization of the overall process is still not entirely satisfactory. Just as the scientific requirements for the analytical adaptation of the antigen-antibody reaction have now been met, it remains to be hoped these problems of mechanization will also be rapidly solved. Depending on the concentration of analytes in the test material, simple methods such as radial immunodiffusion, or mechanized methods such as nephelometry (kinetic or end point methods), or turbidity can be used. For trace concentrations, the PACIA method represents an alternative to known and proved, but not problem-free techniques (RIA and EIA). For obvious reasons, a reduction in the number of radioimmunoassays is desirable.

Appendix

Suppliers of reagents and apparatus

Radial immunodiffusion

1. Behringwerke AG Postfach 1130, D-3550 Marburg/L.
2. Cooper Biomedical Rebstöcker Str. 33–39, D-6000 Frankfurt M.
3. Kallestad Diagnostica GmbH, Colombistr. 27, D-7800 Freiburg.
4. Miles GmbH, Lyoner Str. 32, D-6000 Frankfurt/M.

Nephelometry and turbidimetry

A = apparatus; R = reagents

1. Atlantic Antibodies, Vertrieb durch AHS-Deutschland GmbH. Lerchenstr. 5, D-8000 München 50 R
2. Baker Instruments, Vertrieb durch Fa. Vogel GmbH, Marburger Str. D-6300 Gießen A, R
3. Beckman Instruments GmbH, Frankfurter Ring 115, D-8000 München 40 A, R
4. Behringwerke GmbH, Postfach 1130, D-3500 Marburg/L. A, R
5. Boehringer Mannheim GmbH. Sandhofer Str. 116, D-6800 Mannheim 31 A, R
6. Cooper Biomedical, Rebstöcker Str. 33–39, D-6000 Frankfurt/M. ehem. Travenol GmbH, Sparte Hyland Diagnostica. A, R
7. DAKO, Vertrieb d. Fa. Boehringer Ingelheim Diagnostica GmbH Gutenbergstr. 3, D-8046 Garching/München R
8. Immuno GmbH. Postfach 103080, D-6900 Heidelberg A, R
9. Kallestad Diagnostica GmbH, Colombistr. 27, D-7800 Freiburg i.Br. R

Nephelometric inhibition technique

1. Beckman Instruments GmbH, Frankfurter Ring 115, D-8000 München 40 A, R

PACIA technique

1. ACADE S. A. 17, Passage de la Vecquée, Box 4100, B-1200 Brussels
2. TECHNICON GmbH, Im Rosengarten 11, D-6368 Bad Vilbel A, R

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An Evaluation of Immunological Methods Based on the Requirements of the Clinical Chemist[1])

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(Received December 27, 1983)

Summary: The practicability, operation time and cost of ligand binding assays are clearly more favourable than those of the possible and sometimes highly specific alternatives. At the same time, however, they have distinct weaknesses with respect to the criteria of analytical performance. For the routine determination of individual clinical chemical parameters, which are otherwise difficult to quantify, ligand binding assays (especially in those versions that do not use radioisotopes) are markedly superior to other methods. Chromatographic methods, however, offer greater advantages for the determination of profiles.

Wertigkeit immunologischer Methoden aus der Sicht des Klinischen Chemikers

Zusammenfassung: Der Vergleich von Ligandenassays mit ihren möglichen, teilweise hochspezifischen Alternativen zeigt ihre klare Überlegenheit bezüglich der Praktikabilität, Arbeitszeit und Kosten. Gleichzeitig werden aber auch ihre Schwächen hinsichtlich der analytischen Methodenkriterien deutlich. Zur routinemäßigen Bestimmung von sonst schwierig quantifizierbaren klinisch-chemischen Einzelkenngrößen sind Ligandenassays, vor allem in ihrer Variante als Nicht-radioisotopenassays den anderen Verfahren eindeutig überlegen, zur Bestimmung von Profilen allerdings den chromatographischen Methoden eindeutig unterlegen.

Introduction

When ligand binding assays were introduced in 1960 by *Yalow & Berson* and by *Ekins* (1, 2) the epoch-making importance of this technique and its subsequent far reaching application were not foreseen. Owing to the esoteric nature of the procedure, it was used only by a few initiates. With the advent of its commercialization in 1975, the method became widely available, albeit chiefly in the form of the radioimmunoassay. Nowdays the technique has widespread application, it is no longer considered to be special or exceptional, and it can now be evaluated dispassionately.

For the laboratory clinician and the clinical chemist, ligand binding assays represent only one of several groups of methods. They must therefore address themselves directly to the problem of comparing ligand binding with alternative methodology. Such a comparison is attempted in the present paper, and the subject is viewed from the standpoint of those who must use these methods daily.

Several problems have already been touched upon or discussed in previous papers, but in the interests of a balanced presentation repetition cannot be entirely avoided. Since limitations of space make it necessary to be selective, the following discussion will deal largely with the areas of endocrinological diagnosis and drug monitoring.

The decision for or against the introduction of an analytical procedure is dictated fundamentally by the answers to three questions:

1. Does the measured parameter provide an answer to the clinical enquiry?
2. Which method gives optimally useful clinical information?
3. Does the chosen method fulfil the stated requirements with respect to quality of analysis, time response, practicability and cost?

Since questions 1. and 2. are outside the remit of the present discussion, they will not be discussed further.

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

General

As alternatives to the immunoassay (radio-, enzyme-, fluorescence- and bioluminescence-immunoassays) one can consider methods based on electrophoresis (e.g. isoelectric focussing), spectrophotometry, fluorimetry, radioenzymic assays and chromatography, e.g. gas chromatography (GC) or high performance liquid chromatography (HPLC). All these alternative methods are applicable completely or in part to the determination of steroids, iodoamino acids and biogenic amines, but they are not suitable or have only doubtful value for the measurement of peptide- or proteohormones (tab. 1). Pharmaceuticals can be determined with the above methods.

Tab. 1. The suitability of various methods for the determination of hormones, pharmaceuticals, proteins and peptides.

	Immunoassays	Gas chromatography	High performance liquid chromatography	Other (photometric, fluorimetric, radio-enzymic) methods
Hormones				
Steroids	+	+	(+)	+
Iodoamino acids	+	(+)	(+)	(+)
Biogenic amines	–	+	+	+
Peptide hormones	+	(+)	?	–
Proteohormones	+	–	?	–
Pharmaceuticals	+	+	+	+
Proteins, Peptides (antigens, antibodies, enzymes and others)	+	–	?	+

Gas chromatography and gas chromatography-mass spectrometry (GC-MS) have the following limitations. Since the material to be measured must be volatile, these methods are normally suitable only for substances of molecular weight less than 1000. Polar and unstable compounds must be derivatized to decrease adsorption or increase stability.

Comparison of immunological methods with the alternatives on the basis of the fundamental analytical steps (tab. 2) shows that the reaction step, the separation step after the reaction (with the exception of the homogeneous techniques) and the detection are all absolutely necessary in the immunological procedure. In the chemical methods only the detection is indispensable, the separation and reaction steps being optional.

Tab. 2. Fundamental analytical steps in ligand binding assays and "chemical" methods.

Analytical step	Ligand binding assays		"Chemical" methods
	homo-geneous	hetero-geneous	
Separation step(s)	(+)	(+)	(+)
Reaction	+	+	(+)
Separation step(s)	–	+	(+)
Detection	+	+	+

During the reaction stage of ligand binding assays, comparatively weak intermolecular interactions are involved, such as the formation of hydrophobic or hydrogen bonds. In contrast, derivatization involves the formation of covalent bonds, which are naturally more stable, and resistant to interference.

Quality

Since the value of immunological methods from the standpoint of the clinician has been presented separately (3), I can limit myself here to a discussion of the quality of the analysis, and omit the diagnostic aspects.

A comparison of the analytical performance of the immunoassay, gas chromatography, gas chromatography-mass spectrometry and the bioassay (tab. 3) shows that gas chromatography-mass spectrometry is superior on all counts, providing the analyte is amenable to this procedure.

Tab. 3. Comparison of the analytical performance of immunoassay, gas chromatography-mass spectrometry and bioassay.

	Immuno-assay	Gas-chromato-graphy-mass spectrometry	Bioassay
Accuracy	(+)	+	(+)
Precision	5–15%	1–5%	10–30%
Specificity	(+)	+	(+)
Detection limit	10^{-12}	10^{-14}	10^{-10}
Assay range	1:50	$1:10^4$	1:20
Linearity	–	+	–

Specificity and accuracy

In the absence of a signal, it can be assumed with a high degree of certainty that the analyte is not present. It is less certain that a positive signal is due to the substance in question.

As shown in figure 1, reputedly highly specific immunological methods may sometimes be subject to a certain lack of specificity. *Voelkel & Tashjian* investigated calcitonin in urine (4) and found differences, which were sometimes very pronounced, between the biologically and immunologically determined concentrations. Such deviations frequently show no definite relationship to each other, so they cannot be predicted or corrected by calculation. A further example is the determination of ocytocin after incubation with pregnancy serum (fig. 2) (5). In this case, the values determined radioimmunologically were much higher than those from the bioassay. A similar situation exists for angiotensin II and insulin.

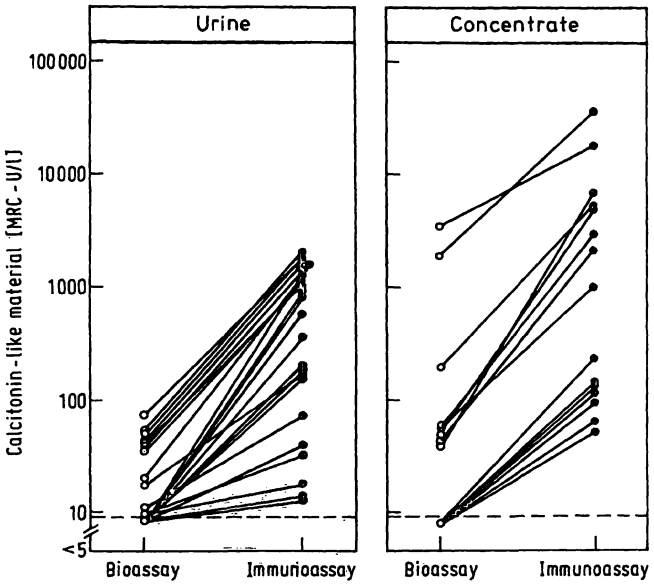

Fig. 1. Calcitonin concentration in urine and urine concentrate measured by bio- or radioimmuno-assay in patients with medullary carcinoma of the thyroid (4).

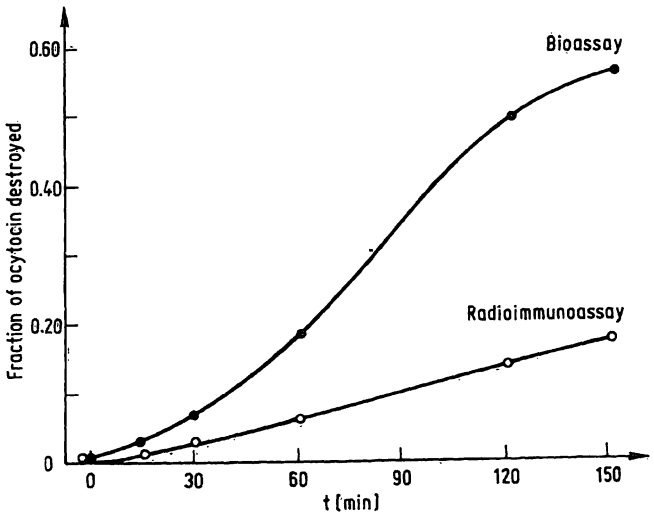

Fig. 2. Decrease in ocytocin concentration by incubation with plasma from women during late pregnancy, measured by bio- (●—●) and radioimmuno-assay (○—○) (5).

Hormone standards from different species may possess the same biological activity, but different immunoreactivity. Conversely, chemically different substances of different biological activity may have similar immunochemical reactivity. Likewise, as already mentioned, fragmentation products, metabolites or prohormones of the compound in question can lead to erroneously high values. The extent of loss by degradation of an analysed substance in the sample solution (frequently serum, which contains enzymes) can be very different from that in the standard solution.

These phenomena can be explained by the presence of either hormone precursors or hormone cleavage products, which have no biological activity, but possess immunological reactivity. It is known that the binding site of an antibody recognizes antigenic determinants equivalent in size to only 4 amino acid residues or 6 hexose residues (6).

Precision

Under routine conditions, the interassay precision of immunological methods usually lies between 5 and 15%. It should be mentioned, however, that this imprecision can be decreased by a factor of 1.5—2 under favourable circumstances, e.g. in a laboratory test study, with equivalent charges, continuity of personel and low pressure of work (7).

Series to series precision is essentially similar for mechanized EIA (thyroxin), mechanized RIA (cortisol) and manual RIA (thyrotropin). Only the determination of oestradiol, which involves a solvent extraction, shows a distinctly higher variation coefficient (tab. 4).

Tab. 4. Inter-assay precision for mechanized EIA (thyroxin), mechanized RIA (cortisol), manual RIA (thyrotropin) and manual RIA with solvent extraction (oestradiol).

	$\bar{x}$	CV (%)
Thyroxin (µg/l)	33	13.9
	92	4.3
	167	4.0
Cortisol (µg/l)	114	8.6
	233	7.3
	310	5.2
Thyrotropin (mU/l)	2.4	9.2
	12.0	4.5
Oestradiol (ng/l)	120	14.6
	260	12.4
	479	10.8

In contrast to the immunological methods, the precision of the gas chromatography-mass fragmentographic methods with internal standardization lies between 1 and 5%.

Three examples serve to demonstrate the precision and accuracy of interlaboratory investigations: aldosterone, which can be standardized with a highly accurate reference method; total T_4, for which there is a defined standard material, but no reference method; and follitropin, as an illustration of a method of unknown accuracy, calibrated against a standard preparation (e.g. MRC 68/40). Surprisingly, the medians of the results obtained with various commercial or self-prepared reagent kits showed about the same maximal scatter (factor of 1.4 to 2.5) (tab. 5) for all three parameters. This was considerably increased, however, for the 16th. percentile (8).

Interference

Patients with paraproteinaemia often show a clinically meaningless total T_4 profile. Patient serum may even contain antibodies against the investigated antigen or against the antibody of the assay, thus rendering the analytical results useless.

Owing to the weak nature of the intermolecular forces in the immune reaction, alterations of pH, ionic strength, temperature and matrix exert a large influence on the results.

Detection limit

The maximal detection limits of different analytical procedures are shown in figure 3, and compared with the normally encountered serum concentrations of various parameters. Haemoglobin is included as the blood analyte with the highest concentration, while substances such as triiodothyronine (T_3), parathyrin, aldosterone, corticotropin and oestradiol are present in especially low concentrations. Most pharmaceuticals are found in the concentration range 10^{-2} to 10^{-1} g/l. To give a mental picture of the detection limits that can be achieved nowdays, let us consider 1 g as equivalent to the distance between the earth and the moon (about 400000 km); 1 pg than corresponds to the dot of a letter i (0.4 mm). Such quantities can be measured by GC-MS with a precision decidedly better than 10%.

Linearity

Reference curves for immunological assays are nonlinear. This means that the distribution of errors in the assay range is not homogeneous. Usually only the first quarter of the reference curve has a steep slope. At higher concentrations the curve becomes increasingly flat. The assay range of 1:60 for thyrotropin is relatively high for a radioimmunological method. The range of 1:6 for valproic acid is more typical. In contrast, the linearity and assay range for gas chromatographic-mass spectrophotometric methods are frequently more than 1:1000.

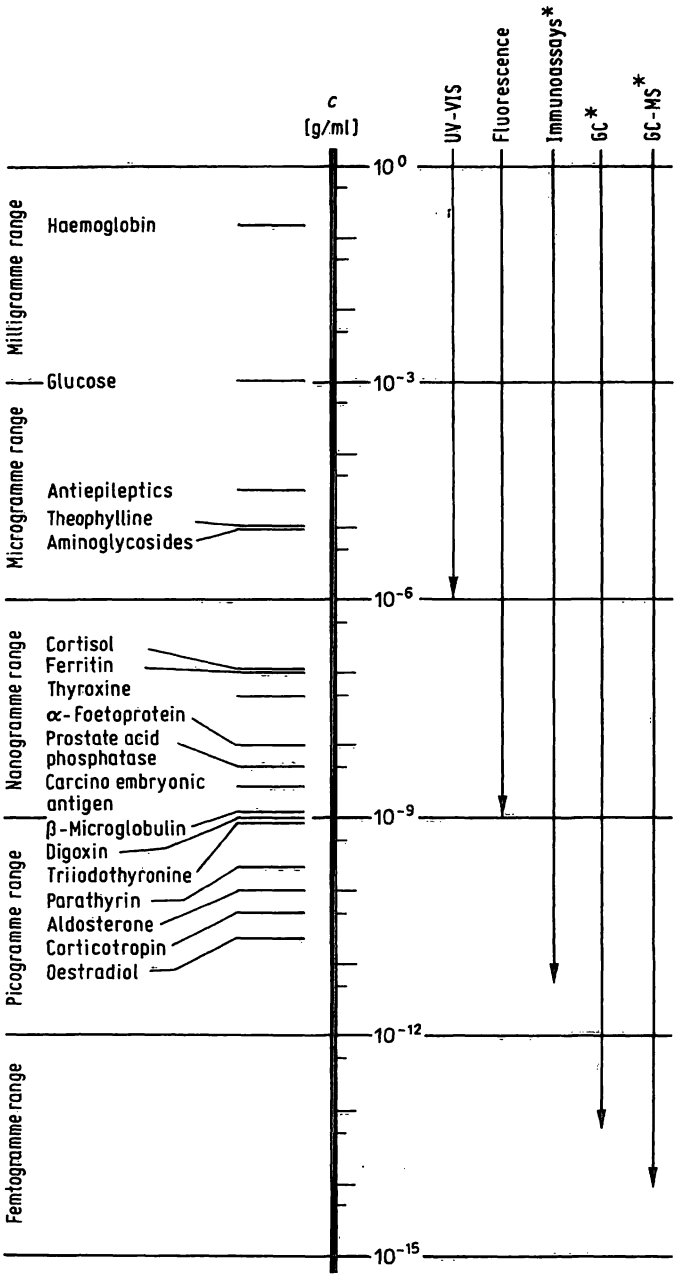

Fig. 3. Comparison of detection limits attainable for analytical methods and typical concentration of various clinical chemical analytes. Diverging from the rules of the Système International d'Unités g/ml was chosen as the unit of substance concentration, as only this allows for a meaningful synopsis. For procedures marked with a star, the substance amounts detectable absolutely are given.

Tab. 5. Scatter of values in an interlaboratory survey of hormone determinations organized by the German Society for Clinical Chemistry 1/83 (8). In each case the maximal (——) and minimal (– – –) value are indicated for the 16th., 50th. and 84th. percentiles, when the number of users of a method was greater than five.

Aldosterone (nmol/l)							Total thyroxin (nmol/l)							Follitropin (U/l)						
KIT	N	MIN	16P	50P	84P	MAX	KIT	N	MIN	16P	50P	84P	MAX	KIT	N	MIN	16P	50P	84P	MAX
AL	46	0.038	0.247	0.4085	0.5754	1.01	AL	207	7.1	79	92.9	108	797.2	AL	133	1.7	5.526	7.5	10.75	20
04	6	0.39	0.4	0.49	0.7701	0.795	04	7	77.4	81.22	95.5	106.6	108	08	22	1.7	5.74	6.7	7.3	7.7
44	6	0.08	0.0844	0.3975	0.5445	0.56	08	13	8.772	76.88	93.4	103.6	125.1	18	1	18.9		18.9		18.9
52	9	0.038	0.1154	0.393	0.4307	0.46	12	5	77.4		82.56		83.9	20	8	6.5	6.757	8.3	9.344	9.9
53	2	0.45		0.5		0.55	16	2	96.9		105.9		115	21	4	5		5.65		6.1
54	1	0.35		0.35		0.35	18	16	50.1	88.32	96.73	108	129	28	1	7		7		7
62	1	0.42		0.42		0.42	20	9	75.47	77.29	93.53	126	158.7	32	9	4.26	4.518	5	5.083	5.2
64	1	0.47		0.47		0.47	24	4	85.1		100		116	44	15	3.2	7.748	8.4	16.03	20
77	11	0.26	0.3504	0.526	0.728	1.01	29	24	67	68.36	74.9	87.25	797.2	52	6	6.7	6.773	9	10.18	10.2
98	1	0.82		0.82		0.82	32	19	79.7	82.64	88	94.09	98	53	10	9.9	10.42	11	13.76	14.8
99	8	0.165	0.189	0.352	0.5458	0.58	36	18	90.2	97.01	100.3	110	114.2	76	33	4.2	6.243	7.3	9.361	11.3
							40	28	12.73	89.6	107.4	116.5	165.2	77	7	8.2	8.2	8.9	12.76	14
							44	17	80.13	87.38	93.7	111	113.5	98	7	4.3	4.569	12.5	17.85	19
							46	3	84		93.48		108.2	99	10	5.3	5.3	7.2	13.51	15
							48	10	79.7	80.89	86.41	92.65	97.46							
							52	1	103.2		103.2		103.2							
							56	1	100.6		100.6		100.6							
							61	5	69.3		79		91.6							
							76	6	7.1	15.6	86	95.85	96.37							
							98	9	74	77.52	90.8	99.8	104.5							
							99	10	69	78.69	92.5	113.9	137							
Quotient $\frac{max}{min}$			4.7	1.5	1.8					6.2	1.4	1.4					2.3	2.5	2.4	

Duration

Comparison of the pre-analysis and analysis times for a simple and an involved procedure shows that in each case immunological methods are distinctly superior to gas chromatography (fig. 4). The total analysis time for the determination of the steroid profile of a urine sample is nearly 90 hours, including 22 hours of pre-analysis operations. By comparison, the 27 hours needed for the total analysis of thyrotropin by RIA is short, despite the fact that this particular procedure is known to be rather time consuming compared with other radioimmunoassays. A similar relationship is evident from the comparison of the two simpler analyses.

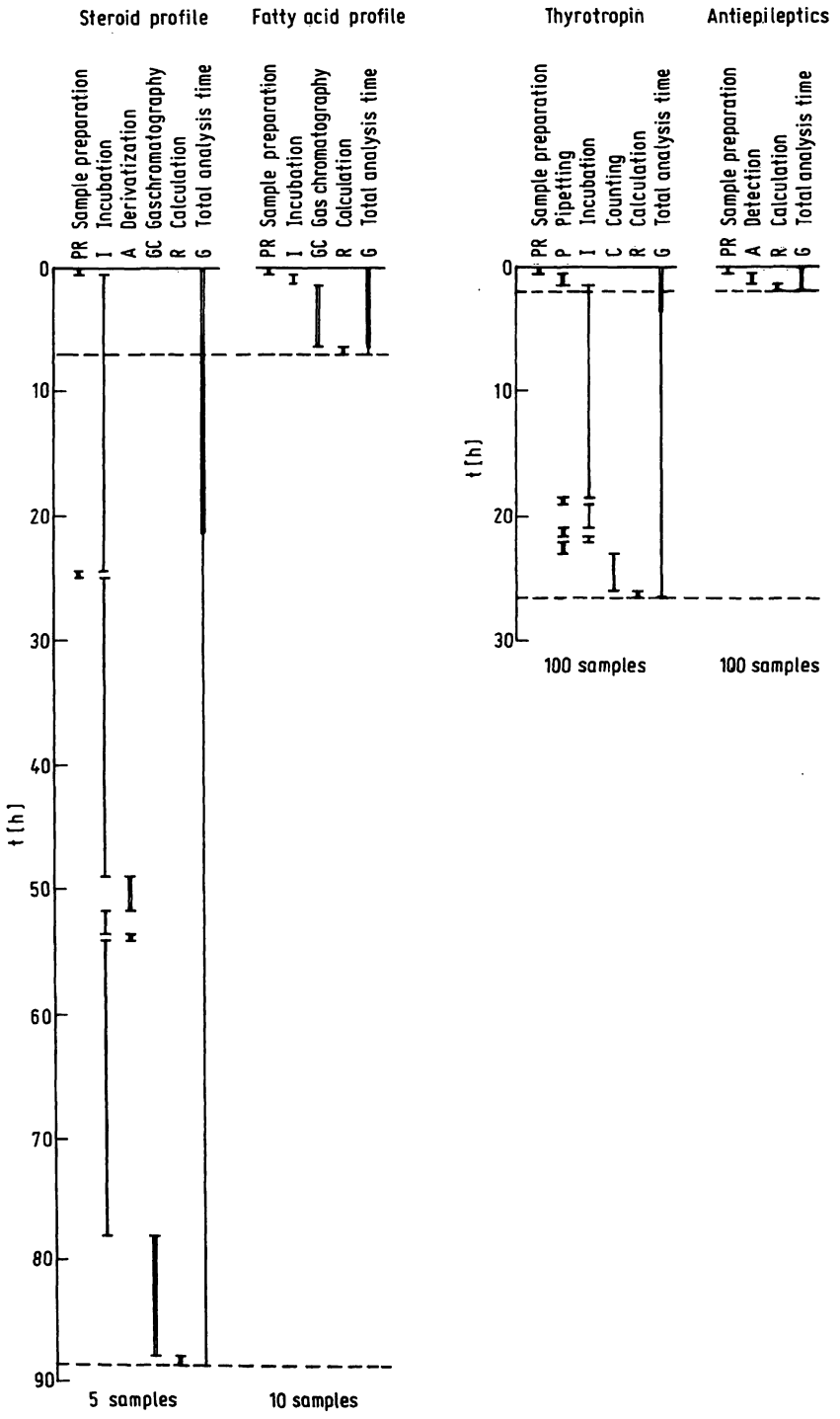

Fig. 4. Bench work (▬) and analysis (——) times for two gas chromatographic methods (steroid and fatty acid profiles) and two ligand binding assays (thyrotropin by manual radioimmunoassay, and antiepileptic agents by a fully mechanized, homogeneous enzyme-immunoassay).

Practicability

Table 6 shows the number of ligand binding assays against the actual number of requested analyses. This ratio is always greater than two, because duplicate determinations are normally performed. It depends strongly on the number of points required on the reference curve, and the number of control samples used. In addition, a series may be repeated if the initial data are of poor quality. Thus, for thyrotropin, a ratio of determinations to requested analyses of 4 indicates that an average of four determinations must be performed to obtain one result. Short series, e.g. valproic acid assays, show an even less favourable ratio.

Tab. 6. Ratio of the number of analyses to the number of requested investigations.

	U	P	U/P	Method
Diphenylhydantoin	3018	744	4.1	EIA-Cobas
Valproic acid	1950	282	6.9	EIA-Cobas
Digoxin	18240	5658	3.2	EIA-LKB
Thyroxin	27642	9894	2.8	EIA-LKB
Triiodothyronine	25800	9917	2.6	RIA-C 4
Thyrotropin	14916	3696	4.0	RIA-man

U = number of analyses
P = number of requested investigations (figures for 1983)
Cobas = CobasBio, Hoffmann-La Roche
LKB = Ultralab system 2074
C 4 = Concept-4, Micromedic
man = manual, Henning

At this point we cannot ignore the fact that the care and disposal of radioactive materials has become an increasingly difficult problem in recent years. Reassuring statements that the disposal of radioactive waste presents no problems are either euphemistic, or they are based on the non-observance of the statutary regulations. Our institute alone produces each year 18 drums of solid and 8 × 60 litre cannisters of liquid waste, which must be stored for several years in a separately appointed room of the clinic for radioactive decay. In comparison, the management of non-radioactive immunoassays is naturally free from such problems, which is why we fundamentally prefer them.

The requirements for apparatus and space, as well as the qualification of the operator, are exorbitantly higher for gas chromatography-mass spectrometry than for immunoassays.

Costs

In view of present day financial stringencies, the question of costs is especially important. Calculation of the real cost in public services is, of course, extraordinarily difficult. The figures quoted here can only be taken as a guide.

There are various reasons for this. The cost of a BAT (Bundes-Angestellten-Tarif, Federal Employee Tariff) post is calculated simply as the sum of the salary and the social taxes. Figures for overheads (e.g. total administrative services) are not included and are not available. Furthermore, information is lacking on operational costs, building depreciation and the interest on capital.

The net working time, based on a 40 hour week, allowing for holidays, illness, etc., is 100320 minutes per year. This is about the same as quoted in the guidelines for the reagent producing industry. The cost of a BAT Vc appointment (1983 scale) is 47050 German marks, which represents 0.469 mark/working minute. For a BAT Vb appointment, this figure becomes 0.514 mark/working minute.

The provision of apparatus and its depreciation contribute markedly to the total costs.

Assuming a linear depreciation to zero over ten years, the equipment costs per analysis range from 0.2 mark for the manual RIA to 106.25 marks for the computerized combined gas chromatography-mass spectrometry.

The total costs per analysis have been calculated from our figures, and they are presented as four separate examples in tables 7—10. Thus, the average cost of a mechanized enzyme-immunological determination is 5.47 marks, without taking account of building costs and electricity, etc. For a manual RIA, the corresponding figure is 7.34 marks. If the calculation is based on the guidelines for industry, the result is similar, although the figure then includes all costs. Comparison with the corresponding figures for gas chromatography (94.15 marks per analysis) or gas chromatography-mass spectrometry (214.95 marks per analysis) clearly demonstrates that these latter methods cannot be considered as alternatives for routine purposes.

This overall assessment shows that at present there is no reasonable alternative to the ligand binding assay for the measurement of individual compounds that are otherwise difficult to analyse. Of the different types of ligand binding assay, those that do not employ radioisotopes are decidedly preferable. On the other hand, for the determination of several different substances simultaneously, chromatographic techniques are clearly superior to ligand binding assays.

Tab. 7. The cost of one determination by mechanized enzyme-immunoassay. One technical assistant performs 32540 assays per year.

3.1	min/investigation
1.45 DM	technical assistant/investigation
+ 0.41 DM	academic supervision/investigation
1.86 DM	personel costs/investigation
0.21 DM	apparatus costs/investigation
3.40 DM	reagent costs/investigation
5.47 DM	total cost/investigation

Tab. 8. The cost of one determination by manual radioimmunoassay. One technical assistant performs 12330 assays per year.

8.1	min/investigation
3.82 DM	technical assistant/investigation
+ 0.41 DM	academic supervision/investigation
4.23 DM	personel costs/investigation
0.20 DM	apparatus costs/investigation
3.00 DM	reagent costs/investigation
7.43 DM	total cost/investigation

Tab. 9. Average costs of a gas chromatographic determination. One technical assistant performs 1100 assays per year.

91.2	min/investigation
46.50 DM	technical assistant/investigation
+ 39.00 DM	academic supervision/investigation
85.50 DM	personel costs/investigation
3.90 DM	apparatus costs/investigation
5.75 DM	reagent costs/investigation
94.15 DM	total cost/investigation

Tab. 10. Average cost of a gas chromatographic-mass spectrophotometric determination. One technical assistant performs 800 assays per year.

125.4	min/investigation
63.95 DM	technical assistant/investigation
+ 39.00 DM	academic supervision/investigation
102.95 DM	personel costs/investigation
106.25 DM	apparatus costs/investigation
5.75 DM	reagent costs/investigation
214.95 DM	total cost/investigation

(figures for 1983)

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Automated Flow-Cytometric Identification of Colo-Rectal Tumour Cells by Simultaneous DNA, CEA-Antibody and Cell Volume Measurements[1])

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Summary: A new method for the automated flow-cytometric identification of colo-rectal tumour cells was developed. Fresh tissue is cut mechanically to obtain single cell suspensions. The cells are then incubated with antibodies in an indirect immunofluorescence assay for CEA (carcino-embryonic antigen) on the cell surface, and counterstained with the DNA stain propidium iodide. Monosized latex particles are added as internal standard, then cell volume, antibody fluorescence and DNA are measured simultaneously in a FLUVO-METRICELL flow cytometer. A FORTRAN IV computer program was used to determine whether aneuploid cells or cells with high density of CEA on their surface were present in the sample. All relevant data were stored automatically in a self updating data base, which is important for quality control and automated thresholding. The samples were taken from 120 different patients. A tumour sample and a sample of healthy adjacent mucosa of the same patient were available in 88 patients. 97.5% of all tumours and 88.6% of the normal mucosa samples were correctly identified. This shows for the first time that the majority of colo-rectal tumour samples can be identified by a flow cytometric measurement with automated data evaluation. The identification of tumour samples was substantially better when based on the measurement of the three parameters, compared with identification by aneuploidy (59%) or by the CEA antibody alone (91%). It will be possible to automate the measurement of the samples.

Automatische Identifizierung colo-rektaler Tumorzellen mit Hilfe simultaner durchflußcytometrischer Messung von DNA, CEA-Antikörpern und Zellvolumen

Zusammenfassung: Es wurde eine neue Methode für die automatisierte durchflußcytometrische Identifizierung colo-rektaler Tumoren entwickelt. Sie besteht aus der mechanischen Zerkleinerung frischen Gewebes zur Einzelzellsuspension, Inkubation der Zellen mit Antikörpern in einem indirekten Immunfluoreszenzansatz für Zelloberflächen-CEA (carcino-embryonales Antigen), Gegenfärbung mit dem DNA-Farbstoff Propidium-Iodid, Zusatz monodisperser Latex-Partikel als internem Standard und simultaner Messung von Zellvolumen, Antikörper Fluoreszenz und DNA-Fluoreszenz jeder Zelle in einem FLUVO-METRICELL Durchflußcytometer. Mit einem FORTRAN IV Computer-Programm wurde bestimmt, ob in der Probe aneuploide Zellen oder Zellen mit hoher CEA Packungsdichte auf der Zellmembran vorhanden waren. Alle relevanten Daten der Auswertung wurden automatisch in einer selbstergänzenden Datei abgelegt, was für die Qualitätskontrolle und die automatische Schwellensetzung von Wichtigkeit ist. Es wurden Proben von 120 verschiedenen Patienten untersucht. Von 88 Patienten waren sowohl eine Tumorprobe als auch eine Probe der umgebenden, gesunden Darmschleimhaut verfügbar. 97,5% der Tumoren und 88,6% der normalen Mucosa-Proben wurden richtig identifiziert. Dieses Ergebnis zeigt erstmals, daß die Mehrheit colo-rektaler Tumoren mit durchflußcytometrischer Messung und automatischer Datenauswertung identifiziert werden kön-

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

nen. Die Identifizierung der Tumorproben durch die Dreiparametermessung war wesentlich besser als durch Aneuploidie (59%) oder CEA-Antikörper (91%) allein. Die Probenmessung kann in der Zukunft ebenfalls automatisiert werden.

Introduction

The correct identification and characterization of malignant and premalignant cells in smears, effusions or biopsy material is of great importance for tumour diagnosis, therapy and prognosis. The recent advances in flow cytometric instrumentation, the use of microprocessor technology (1), development of flow chambers for automated instruments (2), new staining techniques for the measurement of functional cell parameters in living cells (3–5) and the increasing number of monoclonal and conventional antisera produced against tumour-associated cell surface antigens open new possibilities for an automated identification of tumour cells by flow cytometry. The reexpression of onco-developmental antigens on tumour cells e.g. carcino-embryonic antigen (CEA) (6, 7) or α-foetoprotein (AFP) is of particular interest, because these antigens are biochemically well characterized. The determination of serum CEA-levels for the recognition of tumour relapses is an established clinical procedure (8), and potent antisera are available for this purpose. CEA is secreted by tumour cells and its cellular location can be demonstrated by immune histology using fluorescein-isothiocyanate (FITC) or peroxidase coupled antibodies (9, 10). Although serum CEA is often increased in gastrointestinal tumours, an increase is not seen for all tumours (8). Furthermore, elevated CEA levels may occur in e.g. smokers or during inflammatory disease without the presence of a tumour. CEA is, therefore, not an absolute tumour marker (7, 8). Hitherto, the measurement of FITC-antibody-labeled cells in flow cytometers and cell sorters has been performed mainly in connection with immunological studies for the identification of cellular subpopulations of the immune system. There has been little work on the determination of CEA-antigen in single cell suspensions of solid tumours, due to difficulties of tissue desintegration and preservation of the antigenic determinants on the cell surface.

In addition to changes of the cell surface antigen pattern, malignant transformation is often associated with chromosomal aberrations resulting in measurable cellular aneuploidy. The flow-cytometric measurement of DNA distribution curves has become a routine procedure during the last decade (11–14). Although cellular DNA-aneuploidy is a good indicator of malignancy, it is not very useful on its own for cancer prescreening purposes, because not all tumours are aneuploid.

Few reports are available on combined DNA-immunofluorescence measurements for tumour cell recognition. We have shown in an earlier investigation (15) that the simultaneous flow cytometric measurement of DNA, CEA-immunofluorescence and cell volume is a fast and sensitive method for identifying cervix uteri cancer cells in suspensions. The purpose of this study was to extend this work to gastro-intestinal tumours. The study was also undertaken to explore the conditions for the automation of sample measurement and data evaluation.

Material and Methods

Cells

Between 0.05 to 0.5 g tumour tissue was removed from malignant colo-rectal tumours immediately after surgery. Tumours from 120 sequentially operated patients,

Dukes stage (16) A: 20.1%,
B: 34.1%,
C: 34.1%,
D: 11.7%;

histological grading (17):

2.5% well differentiated adenocarcinoma (I);

72.5% moderately differentiated (II);

13.3% moderately to poorly differentiated (II–III);

11.7% poorly differentiated (III).

A second tissue sample of similar size was taken from the healthy adjacent intestinal mucosa of the same patient. Both samples were immersed in a 0.15 mol/l NaCl solution, buffered with 10 mmol/l Tris/HCl to pH 7.35 (Tris buffered saline), cooled to 0 °C in an ice bath and kept at 0–4 °C during the following procedure until cell fixation. The samples were separately minced once with a *McIlwain* electric tissue chopper (The Mickle Comp., Gomshall, England). The chopper was modified so that five parallel razor blades with 0.8 mm spacing were used as the cutting knife instead of the one blade provided by the manufacturer. The chopped tissue was taken up in 10 ml Tris buffered saline containing 10 mmol/l EDTA in a 50 ml plastic Falcon tube with conical bottom (Becton Dickinson, Heidelberg, FRG). The suspension with the small tissue pieces was quickly sucked 30 to 50 times back and forth in a 1 ml Eppendorf pipette (Eppendorf, Hamburg, FRG) without producing air bubbles. The disposable plastic tip of the pipette was cut for this purpose at the bottom to obtain an opening of 0.5 or 1 mm in diameter in order to let the tissue pieces just freely pass. The cell suspension was filtered through a V2A-steel sieve of 60 µm mesh width, washed twice by centrifugation in 50 ml Tris buffered saline at 300 *g* and resuspended in Tris buffered saline at a cell concentration of 5×10^7/ml. The majority of the cell suspension was fixed at 0 °C for 12 h by addition of an equal amount of a freshly prepared formaldehyde solution (35 g/l Tris buffered saline adjusted to pH 7.35). For immunofluorescence staining, the fixed cells were washed twice with 50 ml Tris buffered saline to eliminate the formaldehyde and resuspended at a cell concentration of 5×10^7/ml in Tris buffered saline containing 15 g/l bovine serum albumin and 40 mg/l of propodium iodide (Sigma Chemicals, Munich, FRG) to stain the cellular DNA. The remaining ali-

quot of fresh cells was centrifuged for 5 min at 300 *g*, resuspended to the original cell concentration in albumin/Tris buffered saline and stained according to the same protocol as fixed cells. 85% of the samples were stained as fixed cells and the remainder as fresh, unfixed cell preparation.

Immunofluorescence

Aliquots (50 µl) of fixed or vital cell suspension were incubated for 12 h at 0 °C in 96 well microtiter plates (250 µl wells), either with 10 µl of an antibody dilution in albumin/Tris buffered saline or with albumin/Tris buffered saline alone as a control. Diluted (1/100) mouse monoclonal anti-CEA antibody (Hybritech, Paesel, Frankfurt, FRG. 1 g/l IgG), or diluted (1/100) rabbit-anti-CEA monospecific antiserum (DAKO, Hamburg, FRG) rendered free of anti-NCA (non specific cross reacting antigen) antibodies by absorption with human spleen and lung tissue (serum kindly provided by Dr. *Lamerz*, Klinikum Großhadern, Munich, FRG, 5.3 g/l IgG) were used to detect cell surface CEA. The assays were washed twice by centrifugation (10 min, 300 *g*) with 200 µl albumin/Tris buffered saline and reincubated with either a fluoresceine isothiocyanate (FITC)-labelled rabbit-anti-mouse or a FITC-labelled goat-antirabbit IgG (both antisera: Paesel, Frankfurt, FRG, 10 g/l IgG, fluoresceine/protein ratio 3.71 (mg/g) and 12.0 g/l IgG, fluoresceine/protein ratio 3.95 (mg/g)) for another 12 h at 0 °C. Both antisera were 1/40 diluted with albumin/Tris buffered saline. The cells were washed twice with albumin/Tris buffered saline and finally resuspended in 200 µl Tris buffered saline containing 40 mg/l propidium iodide. Resuspension of the cells after each wash was effected by a Titertek vibration mixer (Flow-Laboratories, Großmeckenheim, FRG). A sample (5 µl) of porous, NH_2 group-containing, monosized (6 µm diam.) latex particles (kindly provided by Prof. *J. Ugelstad*, SINTEF and University of Trondheim, Norway) were prestained with FITC and added at a final concentration of 2.5×10^5/ml to each assay as internal standard.

Flow-cytometric measurement

The cell volume and the green FITC and the red propidium iodide fluorescence of the double stained cells were determined in a FLUVO-METRICELL flow cytometer (18). The volume of each cell was measured electrically at a current of 0.47 mA in a Tris buffered saline-filled measuring chamber at 22 °C after hydrodynamic focusing of the sample beam through the center of a cylindrical orifice of 85 µm diameter and 100 µm length. The fluorescence was excited by epi-illumination with UV light between 418 and 500 nm from a HBO-100 high pressure mercury arc lamp, after the cell had left the sizing orifice. The red and green fluorescences were separated by a 530 nm dichroic mirror. The green FITC-fluorescence was collected between 500 and 530 nm and the red propidium iodide fluorescence between 550 and 650 nm. The maximum height of each signal was amplified by a 2.5 decade logarithmic amplifier for the cell volume signal and by linear amplifiers for the FITC and propidium iodide signals. The amplified signals of each cell were stored in list mode on magnetic tape and subsequently analysed by FORTRAN IV computer programs described in part earlier (19). The analysis consisted of the calculation and plot of three one parameter distribution curves (cell volume, FITC-antibody and propidium iodide-DNA distribution) (figs. 1, 2), a two parameter FITC-antibody versus propidium iodide-DNA histogram (fig. 4) and a three dimensional cube for visual inspection (fig. 3). The total data evaluation was recently automated. After completion of the measurements, the user only types the word "ANTIGEN" in the computer (INTERDATA 7/32, Perkin Elmer, Oceanport, NJ, USA, 768 kbyte core memory, 200 Mbyte disc, magnetic tape, electrostatic plotter, graphic display) which starts a command-file where all calculation procedures are defined. The user obtains the above mentioned graphs and a data list for each sample. The data list contains the number of antigen positive cells, the mean antigen surface density per cell and the aneuploidy index. The antigen positive cells are the cells beyond a threshold in the FITC-antibody versus propidium iodide-DNA histogram (fig. 4a, b). The antigen surface density of the antigen positive cells was calculated for each cell from the cell volume and the intensity of the FITC-antibody fluorescence, assuming a spheroid shape of the cell. To determine the aneuploidy index 1 (AN1), the product of % cells in $S+G_2/M$ phase times their mean DNA-fluorescence was divided by the product of % cells in G_0/G_1 phase times their mean DNA-fluorescence. The aneuploidy index 2 (AN2) was calculated, when a sample of normal mucosa from the same patient was also available. AN2 is the ratio of AN1 of the tumour divided by AN1 of the mucosa. AN2 was a very sensitive indicator of abnormalities because it indicated minor differences between tumour and mucosa. A sample was judged aneuploid above an AN1 or AN2 of 1.2. All calculated data were automatically stored in a self updating data base which was installed for long time quality control and automated optimation of the decision thresholds. A cell sample was automatically judged malignant by the computer classifier program either when it was aneuploid or when the surface density of CEA-positive cells exceded the normal range.

Results

The identification of samples containing tumour cells was easily possible when aneuploid cells were present. They were visible as a separate peak (fig. 1a) which did not occur in cell samples from the healthy adjacent mucosa (fig. 1b). 71 out of 120 malignant tumour samples (59%) were aneuploid with DNA-indices mostly in the hyperdiploid region (1.15–1.90).

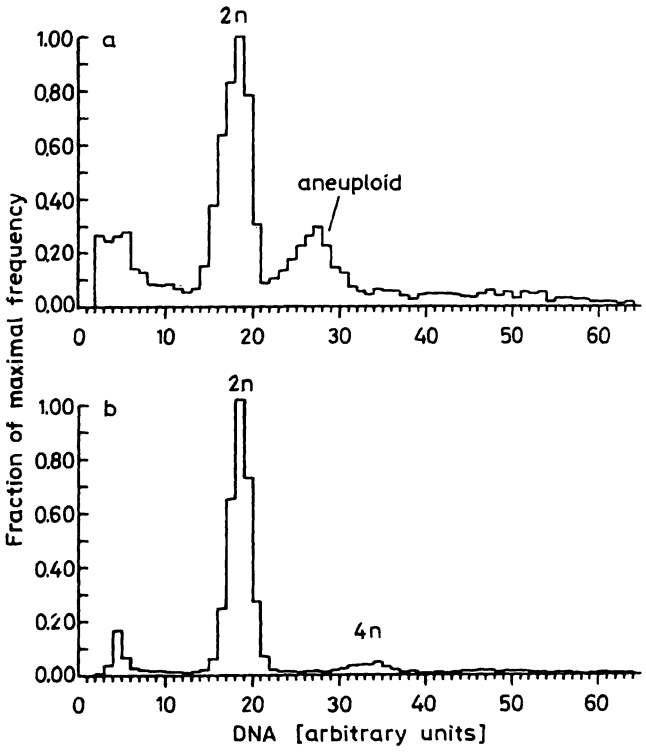

Fig. 1. DNA distribution curve.
a) of an aneuploid human rectum cancer cell sample.
Number of particles measured: 3401.
Maximal frequency: 394.
b) of the adjacent healthy mucosa.
Number of particles measured: 8870.
Maximal frequency: 2243.

The identification of euploid tumour cells was not possible with one-parameter measurements alone. This is apparent from figure 2 where the DNA distribution (fig. 2a, b), the antibody distribution (fig. 2c, d) and the volume distribution curves (fig. 2d, e) of cells of the tumour (fig. 2a, c, d) and the normal mucosa (fig. 2b, d, e) of a patient with a euploid tumour are shown. All curves begin with an exponentially decreasing background in the left part. The respective curves of tumour sample and normal mucosa were quite similar. The shape of the diploid peak of the DNA distributions (fig. 2a, b) was broad with coefficients of variation (CV = 100 · standard deviation/mean value) in the order of 8 to 10%. The CV of the DNA distribution curves was lower (6 to 8%) for fresh cells. The high CV's were due to the intestinal cells and to formaldehyde fixation, but not to instrumental variation because CV's of 1.5 to 1.9% were obtained for ethanol-fixed rat thymocytes. Ethanol fixation caused, however, cell clumping. The diploid peaks of the DNA distribution curves were often left skewed after formaldehyde fixation because the large epithelial cells stained slightly higher with propidium iodide as compared to the

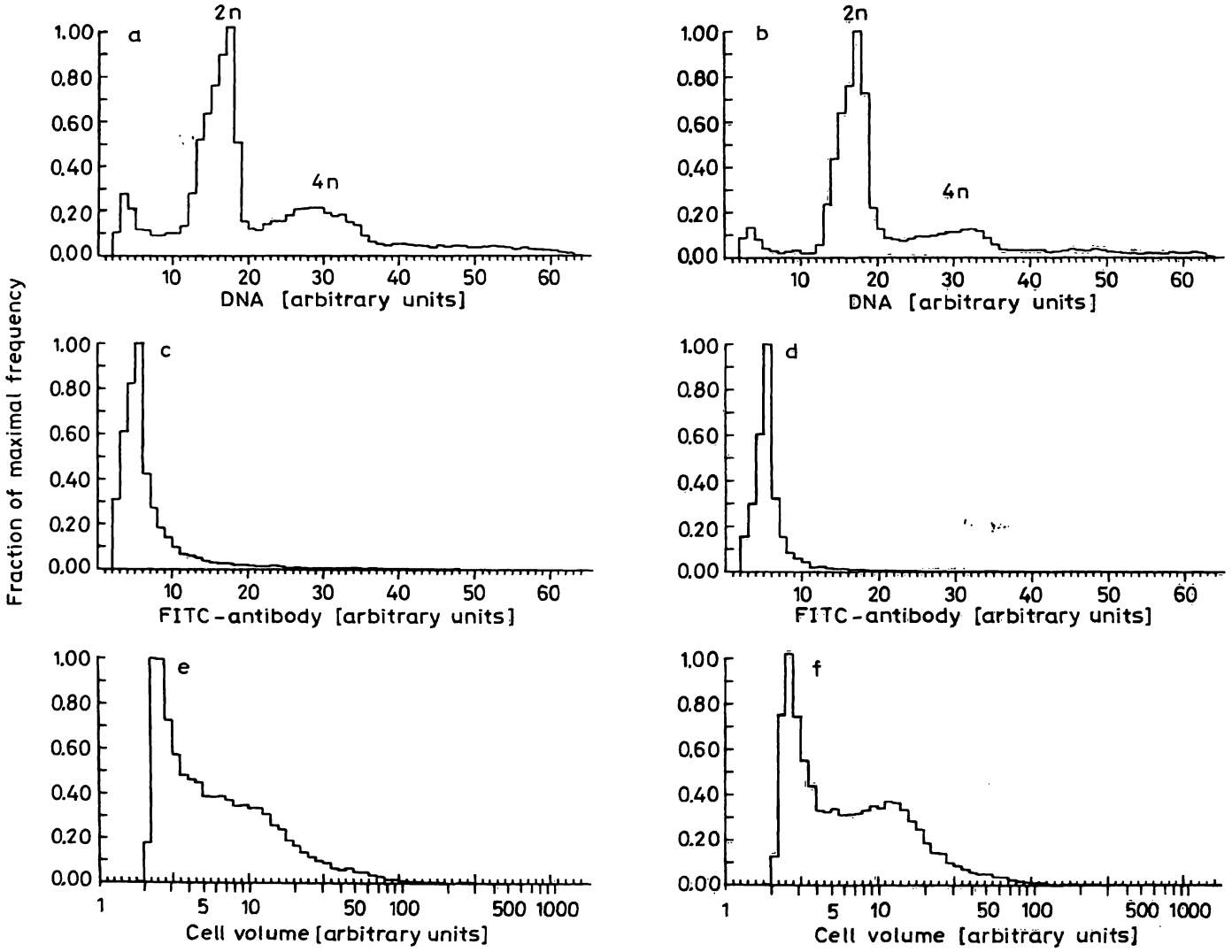

Fig. 2. DNA, FITC-antibody and cell volume distribution of an euploid colon cancer (left) and of the adjacent healthy mucosa (right). All three curves begin with an exponentially decreasing experimental background on the left side of the plot. It is most pronounced in the antibody (c, d) and cell volume distribution (fig. 3e, f).
Between 10600 and 18700 cells per curve were measured.
a) DNA, euploid colon cancer; maximal frequency 1906.
b) DNA, adjacent healthy mucosa; maximal frequency 1547.
c) FITC-antibody, euploid colon cancer; maximal frequency 6286.
d) FITC-antibody, adjacent healthy mucosa; maximal frequency 9496.
e) Cell volume, euploid colon cancer; maximal frequency 2969. One class on the logarithmic volume scale corresponds to 30 fl.
f) Cell volume, adjacent healthy mucosa; maximal frequency 3120.

small inflammatory leukocytes. The left skew is visible in figure 2a, b. Since the identification of euploid tumour cells by the one parameter measurements of figure 2 was not possible one could conclude that flow cytometry is not a suitable method for this purpose.

This was, however, not true, when a simultaneous measurement of the same three parameters was performed. The measurement resolved additional features of the different cell populations (fig. 3a, b). The three parameter measurement distinguishes between cell debris and morphologically intact cells. Only particles with DNA and volume are morphologically intact cells. Cell debris i.e. enucleated cells or broken cells give rise to volume signals but not to DNA signals, and bare nuclei show DNA signals but only very small volume signals which do not appear on the volume scale. Cell debris is significantly present in the tumour sample (fig. 3a) and also in the normal mucosa sample (fig. 3b). Intact, antigen positive cells are visible amongst the cells with large volume in the tumour sample (fig. 3a). This is reasonable because tumour cells occur amongst the large epithelial cells. Some CEA-antigen positive cells were also present in the normal mucosa sample of the same patient (fig. 3b). The intact inflammatory cells at the lower edge of the volume scale are not antigen positive, indicating that the antiserum is free of granulocytes that react with NCA-antibodies. The cluster of calibration particles is also visible. It is situated where no interference with stained cells occurs. The particles serve as internal standard for the calculation of the cell concentration. They are also useful for checking instrument function with regard to cell volume and fluorescence. They allow a precise recalibration of the instrument from day to day, which is important in automated operation for maintaining standard thresholds over a long period of time. The cube display is useful for a quick visual judgment of the three parameter measurement, but not suitable for quantitative evaluation. The quantitative evaluation is performed on two parameter histograms obtained by projecting the content of the cube onto the antibody versus DNA plane (fig. 4a, b). The calibration particles were not projected to simplify the distributions. The solid lines of the graph separate the area of antigen negative and antigen positive cells used by the computer program for the calculation of cell parameters. The substantial amount of cell debris which is strongly positive for the CEA-antibody is again visible. The quantitation would be significantly influenced if the debris were not distinguished from the intact cells by the DNA stain of the cell nuclei. The evaluation of the tumour samples and of the normal mucosa cells of two patients (figs. 1 and 2, 3) is given in table 1. The aneuploidy of the tumour sample of the first patient is

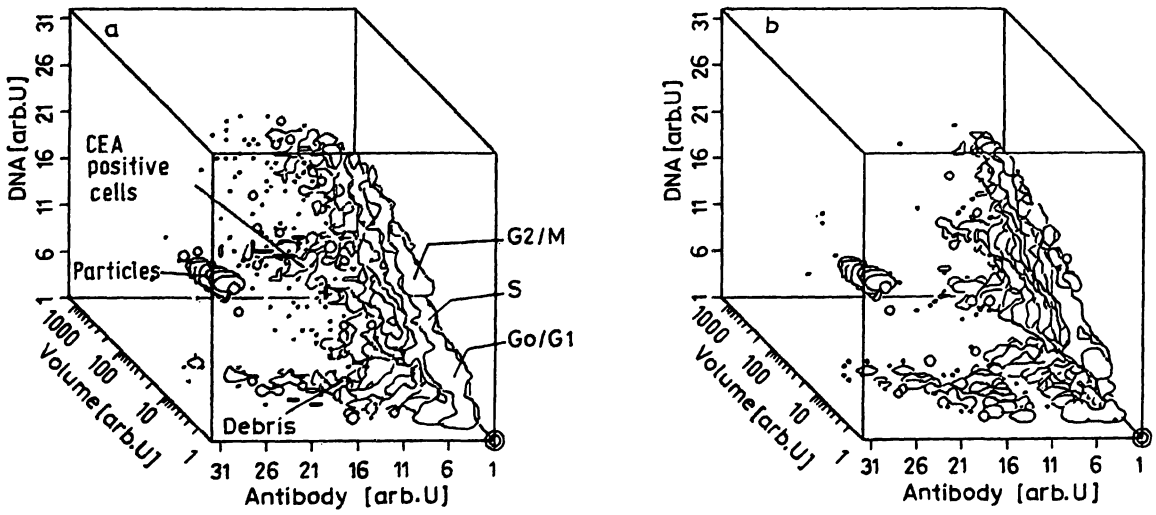

Fig. 3. Three dimensional representation of a simultaneous cell volume, DNA and CEA-antibody measurement of colon cancer cells (a) and cells of the adjacent healthy mucosa (b). The cells are derived from the same samples as those represented in the one parameter distribution curves of figure 2. The increased number of morphologically intact CEA-positive cells in the tumour cell sample (a) is visible. The contour lines surround the areas where particles and cells are located. The channel contents are standardized to the maximum logarithmic channel content and plotted for the 10% level. Channels with 2 and 3 cells are already contoured which means that the location of most of the measured cells is indicated. The calibration particles are used as internal concentration standard and also for day to day standardisation of the fluorescence measurement. One class on the logarithmic volume scale corresponds to 30 fl. 19419 (a) and 11948 particles (b) were measured. The maximum channel contents are 256 and 245 particles respectively. G0/G1, S and G2/M represent the cell cycle phases.

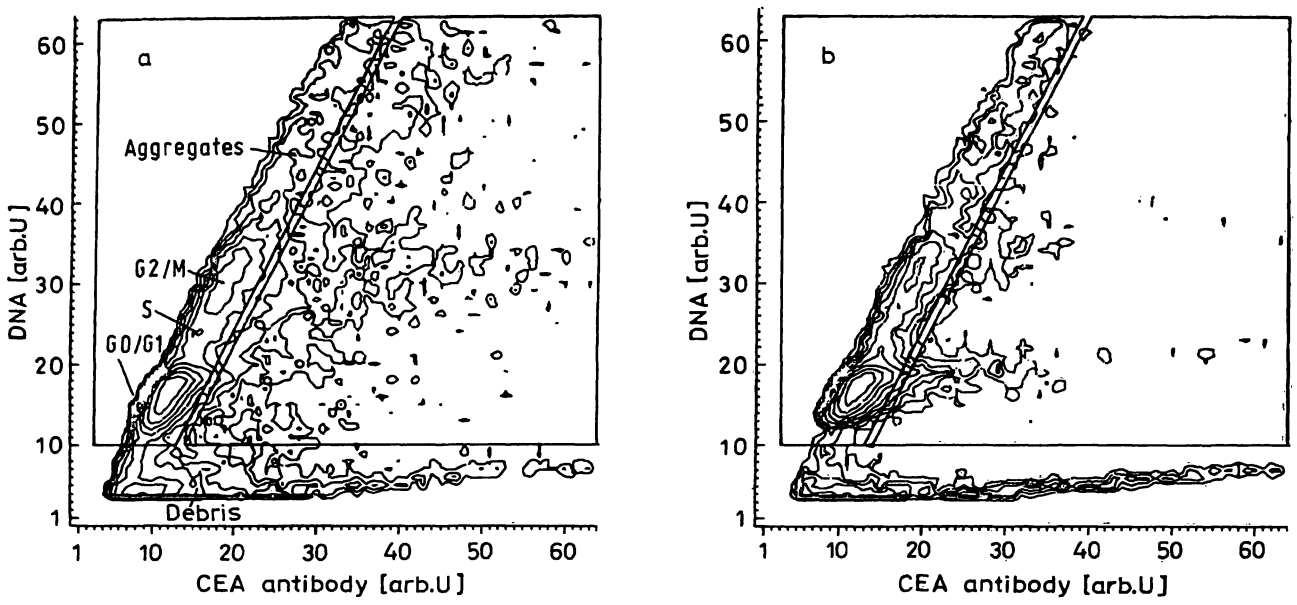

Fig. 4. CEA-antibody versus DNA histogram of colon cancer cells (a) and cells of the adjacent healthy mucosa (b). The graphs were obtained by projecting the respective cube diagrams of figure 3 onto the antibody/DNA plane. The solid lines represent the areas used for the computer evaluation. To the left is the antibody negative and to the right the antibody positive compartment. 16.1% CEA positive cells were found in the tumour sample and 7.9% in the normal mucosa. The graphs were standardized to the maximum logarithmic channel content and contour lines are plotted in 10% linear increments. To simplify the calculation of antibody positive cells the calibration particles of figure 3 were not projected onto the antibody/DNA plane. 18574 (a) and 10665 particles (b) were measured. The maximum channel contents are 523 and 395 particles respectively.

Tab. 1. Calculated parameters from simultaneous CEA-antibody, DNA and cell volume measurement of samples from an aneuploid (fig. 1) and an euploid colo-rectal tumour (fig. 2 to fig. 4).

	CEA-positive cells (%)	Relative antigen density	Aneuploidy index 1	Aneuploidy index 2
Aneuploid				
Rectum carcinoma	15.13	0.653	1.35	3.75
Normal mucosa	4.24	0.406	0.36	–
Euploid				
Colon carcinoma	16.12	0.759	0.842	1.00
Normal mucosa	7.89	0.389	0.840	–

correctly recognized by the AN2 (>1.2). The number of antigen positive cells is abnormally high in both patients and the mean antigen density/cell is substantially increased in the tumour samples. A total of 120 patients with colo-rectal tumours and 88 normal mucosa samples from the same patients have been screened so far by this method. All samples were measurable. The lower number of mucosa samples is due to the fact that some of the tumours were taken from patients prior to cryotherapy without a normal mucosa sample. Also included in the tumour cases are 11 patients from which only metastatic lymph-node material was obtained by abdominal surgery. The mean antigen density on the antigen positive cells of most tumour samples was higher than on the antigen positive cells of the adjacent normal mucosa. 109 (91%) of the tumors were recognized by the CEA-antibody alone. Of the remaining 11 tumours, 8 were aneuploid and thus could be additionally identified. 3 tumours i.e. 2.5% of all tumours were not recognized. The metastatic cells behaved similarly to the tumours. Table 2 contains the quantitative values. The conventional anti-CEA

Tab. 2. Flow-cytometric classification of colorectal tumour and mucosa cells by CEA-antibodies and DNA-aneuploidy.

	Monospecific DAKO-antibody		Monoclonal Hybritech antibody	
	Tumour FN (%)	Mucosa FP (%)	Tumour FN (%)	Mucosa FP (%)
Aneuploidy + % CEA-positive cells	8.3	13.7	6.1	27.5
Aneuploidy + rel. CEA-antigen surface density	2.5	11.4	4.9	18.3

FN = false negative (n = 120 tumour samples)
FP = false positive (n = 88 mucosa samples)

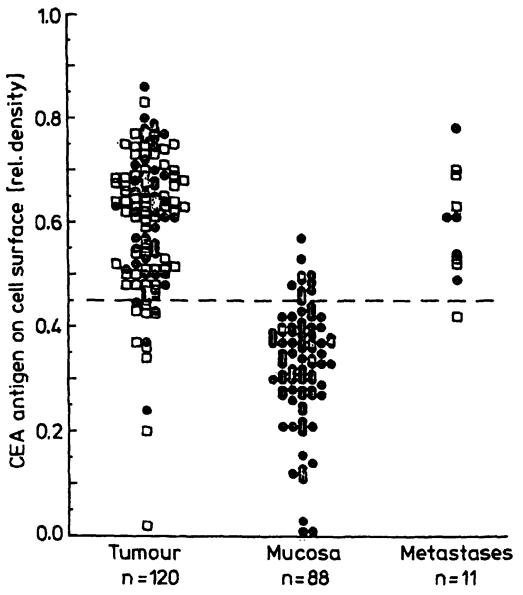

Fig. 5. Mean CEA density on colorectal tumour, mucosa and metastatic cells. The closed symbols represent euploid samples, the open symbols aneuploid tumours. With a fixed borderline at a relative density of 0.45, a fraction of 0.025 of the tumours are false negative and a fraction of 0.114 of the mucosa samples are false positive. For the metastases similar results as for the tumours are obtained.

antibody recognized the cells better than the monoclonal antibody. It is important to note that the recognition of the tumour cell-containing samples was substantially better if the antigen density on the antigen positive cells was compared instead of the number of antigen positive cells alone. Altogether the flow-cytometric method identified correctly 97.5% of the tumours and 88.6% of the mucosa samples in a fully automated way.

Discussion

The most important result of this study is that more than 95% of tumour samples were correctly recognized by an automated flow-cytometric evaluation method. To our knowledge it is the first time that this has been possible. Most flow-cytometric work has been done, so far, on the aneuploidy of tumours. The search for aneuploidy alone is, however, not sufficient for patient screening, since not all tumours are detectably aneuploid (11–14). 59% of the tumours in the present study were aneuploid. This result compares well with values between 50% and 78% given by other investigators (20–22). The measurement of additional cell parameters, such as light scatter and cellular protein (23, 24, 25) do not improve the results very much, because these parameters are not tumour-specific enough.

In the present approach, the first two parameters (cell volume and DNA) were used to identify aneuploid cells, to distinguish the morphologically intact cells from cell debris, and to separate the large epithelial cell compartment with the carcinoma cells (fig. 3a, b) from the small inflammatory cells (lymphocytes, granulocytes). The third parameter, the CEA-antigen of the morphologically intact cells, is important for the recognition of euploid tumour cells. CEA is expressed to a certain extent on normal mucosa cells, mainly on the brush borders, as revealed by light microscopic examination. Although not strictly tumour-specific, the fluorescence signals of the CEA-antibodies shift a significant number of tumour cells into an area of the three parameter cube (fig. 3a, b), where not many cells are located in cases of normal mucosa samples. The cell volume served for calculation of the mean antibody packing density on the cell surface. This is an important aspect of the volume measurement since the cell is used as a minicuvette to obtain standardized data. The validity of such calculations was shown earlier when the electrical charge density on the cell surface of erythrocytes could be correctly predicted by a flow-cytometric measurement (26). It is evident that the simultaneous three parameter measurement offers substantial advantages over one or two parameter measurement.

It can be argued that this method, although superior to other flow cytometric methods is still worse than the normal histo-pathological examination of colorectal tissue sections. This is true at the present time but there are possibilities of improvement. As in the case of the CEA and epithelial membrane antigen (EMA), which are independently expressed on cervix tumour cells (15), it is possible to test antibodies against other tumour associated antigens on colorectal tumour cells (27) for the detection of the remaining 2.5% false negative tumour samples. The automation of a diagnostic flow cytometric procedure is substantially easier than the automated recognition of cancer cells from histological or cytological specimens by computer image analysis. It seems, therefore, of particular interest to further pursue the flow cytometric approach.

There is the practical question of whether automated analytical flow cytometers with automated evaluation can be developed for routine clinical and research application. The operation of a flow cytometer at the present time is comparatively complicated and slow, and typically between 5 and 10 min are required to measure and change a cell sample. The purpose of recent microprocessor (1) and measuring-chamber (2) development was to increase the

speed (30 to 60 s/sample) and simplify the operation of flow cytometers. An important requirement for automation are monosized calibration particles of suitable size, which became available only recently (28). They can be used as internal standard for cell concentration but also for the continuous monitoring of the correct sample flow by the microprocessor data analysis system. The software was adapted for automated operation. The relevant parameters of each measurement are automatically stored in a data bank file which assures the long term stability of the measurements. It will be possible to use automated flow cytometers in the same way as as photometers, thus greatly extending the possibilities for cellular work. In addition to the identification of tumour cells it is, for example, of interest to count blood cells and to determine simultaneously their functional state (29), to measure cellular assays for cytostatic drug testing on patient tumour cells (30), to partially differentiate bone marrow aspirates (31) and to measure radiation damage in bone marrow and blood samples for biological radiation dosimetry (32). Fully microprocessor-controlled flow cytometers are comparatively cheap and will also be accessible to smaller hospitals and to specialized private practice.

Although the prospects for a broader application of flow-cytometry in diagnostic medicine are substantially increasing at the present time, it is obvious that one is still at the beginning of a development. Much further work is needed to elaborate a spectrum of diagnostic methods for cells, comparable to the large number of common clinical chemistry assays. Despite the improvements one has to see the limitations of the method. The tissue architecture has to be destroyed for the preparation of single cell suspensions. Flow cytometry can, therefore, not replace the histopathological examination. Nevertheless, the possibility of performing fast and quantitative biochemistry in single cells is of great interest for the characterization of the alterations of cell metabolism in disease.

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Immunological Procedures in Clinical Enzyme Diagnostics[1])

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Summary: The paper presented deals with the general potentialities of the immunological determination of enzymes, using selected examples to describe the most important variants of the immunoassay for clinical chemical enzyme diagnostics. The principle of each test and the respective possibilities of interference are described. The analytical efficiency and applicabilities of these immunoassays are assessed critically.

Immunologische Verfahren in der klinischen Enzymdiagnostik.

Zusammenfassung: Die vorliegende Arbeit geht auf die grundsätzlichen Möglichkeiten der immunologischen Bestimmung von Enzymen ein und stellt an ausgewählten Beispielen die für die klinisch-chemische Enzymdiagnostik wichtigsten Varianten des Immunoassays dar. Neben einer Beschreibung des jeweiligen Testprinzips und seiner Störmöglichkeiten werden die analytische Leistungsfähigkeit und die Anwendungsmöglichkeiten dieser Immunoassays kritisch beurteilt.

Introduction

Up to now the immunoassay has played only a subordinate role in enzyme diagnostics in the clinical chemical laboratory; here the "classical" photometric test, which determines an enzyme purely by virtue of its function, i.e. its catalytic activity, still dominates.

The immunological determination of enzymes, isoenzymes, proenzymes and enzyme complexes[2]) is however growing increasingly attractive, particularly for the clinical laboratory, due to methodical and technical advances in the field of the immunoassay (e.g. non-radioactive labels, monoclonal antibodies). It is most likely that in the future it will not only replace some of the traditional methods, but also lead to a considerable broadening of the diagnostic spectrum.

Enzymes as Antigens

Due to their protein structure, enzymes represent the prototype of a multivalent antigen. As specific anti-enzyme antibodies react with exactly defined antigenic determinants (epitopes) on the surface of the enzyme molecules, any change in the enzyme conformation, which includes these epitopes, can lead to a change in the antigenicity of the enzyme. Structurally modified enzymes do not only provoke a qualitatively different immune response in the test animal, but they also react differently in the immunoassay.

Frequently, antisera which have been obtained by means of immunization with a particular enzyme also react with structurally similar or related enzyme forms. Such cross reactions are known to occur with proenzymes (1), aged enzymes (2) and enzymatically

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

[2]) Detailed specialized literature is available on request directly from the authors.

silent products of mutated structure genes ("silent genes") (3, 4).

Polyclonal and Monoclonal Anti-Enzyme Antibodies

Even if a highly purified enzyme or isoenzyme is available as an antigen, immunization leads to the formation of anti-enzyme antibodies of varying specificity and affinity. The quality of these conventional i.e. polyclonal antisera can however be improved either by enriching the specific antibodies through affinity chromatography or by removing the unspecific antibodies by means of immune absorption, as shown in the example of a polyclonal antiserum directed against the intestinal isoenzyme of alkaline phosphatase (5).

Monoclonal antisera (6) were introduced for the decisive improvement of the specificity of immunological enzyme assay. It was shown that monoclonal antibodies are even able to differentiate clearly between the very similar allelozymes of alkaline phosphatase from human placenta (7, 8).

Competitive or Non-Competitive Immunoassay

A polyclonal antiserum is best suited for competitive assays such as the classical radioimmunoassay (RIA), as antiserum is diluted to such a high degree in these assays, that only the highly affine i.e. specific antibodies still react with the desired enzyme and the concurrent reaction of unspecific antibodies is to a large extent suppressed. In comparison, in the non-competitive assays, (e.g. immunometric procedures), which work under conditions of an antibody excess, the risk of such undesired cross reactions is considerably greater. It is therefore especially important here to use a qualitatively high-grade i.e. purified polyclonal or monoclonal antiserum. In contrast to the RIA, a labelled enzyme standard is not needed for the non-competitive immunoassay. This is a considerable advantage as radio-iodination can change the immunological reactivity of an enzyme to a high degree. As was shown using the RIA for prostate acid phosphatase for example, this principal source of error can be overcome by the careful selection and control of the radio-iodination technique (9).

Determination of Enzyme by Catalytic Activity or by Mass

In the immune complex, the catalytic activity of an enzyme can be maintained unaltered; however often steric or allosteric effects lead to varying degrees of inhibition but also less frequently to an increase in enzyme activity. In which way and to what extent the catalytic properties of an enzyme are changed by an immune reaction depends primarily on the amount and quality (e.g. specificity, affinity, polyclonal or monoclonal) of the anti-enzyme antibody used. In this connection it is also extremely important whether this antibody reacts with the enzyme in soluble form or – as in a solid-phase immunoassay – in an immobilized form. Moreover, the catalytic activity of an antibody-bound enzyme can be influenced by the size of the substrate. It is known that some enzymes – after binding on the antibodies – convert substrates less effectively, the larger the substrate molecular weight becomes (10, 11). On the other hand, enzymes can be inhibited in their activity considerably more by pre-incubation with the antibody than they are if reaction with the antibody does not occur until after addition of the substrate. Here the substrate quasi "protects" the enzyme from the effect of the antibody (12, 13, 14).

The choice of the type of immunoassay to be used for a particular enzyme depends firstly on the catalytic activity or mass concentration of this enzyme and cross-reacting antigens (e.g. isoenzymes) in the material to be examined and secondly on the precision, speed and practicability required of the assay in reaching its diagnostic objective. Immunoassays which utilize the antigenic as well as the catalytic properties of an enzyme for its quantification are suitable for the assay of enzymes with easily measurable activity. After reaction with the specific anti-enzyme antibodies (separation step) the enzyme is detected via its own catalytic activity. Precipitation, inhibition and adsorption procedures can be distinguished with respect to the separation techniques used. For the measurement of inactive enzyme forms (e.g. proenzymes) these methods are just as unsuitable as they are for enzymes which are quickly inactivated in diagnostically relevant body fluids or whose activity is difficult to measure for technical reasons. Such enzymes can only be quantified by means of their antigenic properties, i.e. their protein mass. For this purpose, besides the RIA, immunometric assays in particular come into consideration.

The most important types of immunoassay which have proved successful in clinical enzyme diagnostics are listed in table 1. They are exemplified and de-

scribed in more detail choosing creatine kinases and phosphatases.

Modifications of the Immunoassay

Immunoprecipitation

In figure 1 the immunological differentiation of the dimerous cytoplasmic creatine kinase isoenzymes by means of precipitating antibodies is shown schematically. In the first step of the reaction, a specific antiserum directed against the M subunit of the enzyme precipitates the isoenzymes MM and MB. The isoenzyme BB remains in the supernatant after centrifugation and can be measured by its catalytic activity. After a second, separate precipitation with an anti-B subunit antiserum, the activity of the creatine kinase-MM is determined. The catalytic activity of the hybrid isoenzyme MB can only be calculated mathematically; it results from the subtraction of the activity of both homomeric isoenzymes from the total creatine kinase activity (15, 16, 17).

The immunoprecipitation renders a selective and quantitative isoenzyme determination possible, if carried out with reliable antibody excess and if the anti-M or anti-B subunit antisera used show no cross reactions with the respective other enzyme subunit. The sensitivity of this method depends on whether the catalytic concentration of the isoenzyme is measured spectrophotometrically (lower detection limit: approx. 3 U/l (25 °C)) or by bioluminescence (lower detection limit: approx. 0.3 U/l (25 °C)) (18, 19, 20).

As the creatine kinase isoenzymes are determined *indirectly*, i.e. by means of the activity remaining in the supernatant, the precision of the method is particularly unsatisfactory in the case of low enzyme activity. Interferences in the immunoprecipitation can be caused by the occurrence of macromolecular creatine kinase variants in the serum (21). These are not precipitated by the antisera (macro-creatine kinase type 2) or are only partially precipitated (macro-creatine kinase type 1). They remain in the supernatant and lead to false results.

Immunoinhibition

By contrast to immunoprecipitation, for which several hours are usually required, the immunoinhibition of enzyme catalytic activity takes only a few minutes. The "CK-MB inhibition test" utilizes this effect (fig. 2). Within 5 minutes an excess of specific

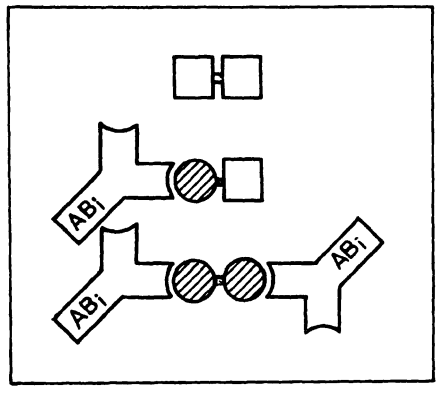

Fig. 2. Determination of the creatine kinase isoenzyme MB by immunoinhibition (22). The catalytic activity of the M subunits is selectively blocked by polyclonal goat antibodies (AB_i), creatine kinase-MB is detected by its residual B subunit catalytic activity.
●: M subunit of creatine kinase (inhibited)
□: B subunit of creatine kinase (active).

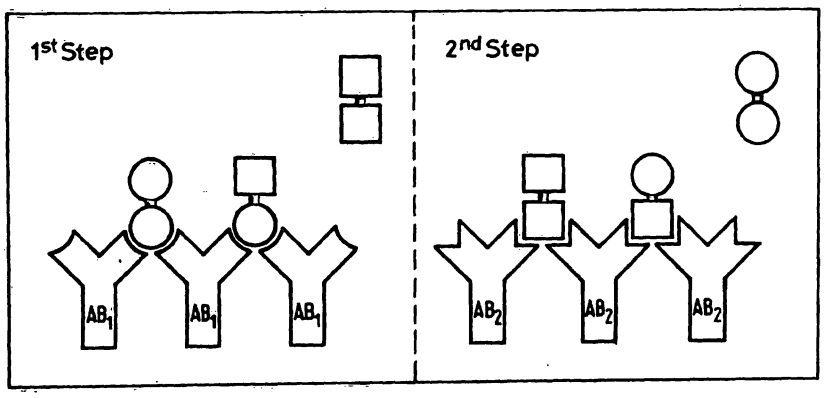

Fig. 1. Differentiation of creatine kinase isoenzymes by immunoprecipitation (17). Catalytic activities of the BB (first step) and MM (second step) isoenzymes are measured in the supernatants; the catalytic concentration of isoenzyme MB can be calculated by the formula:
MB[U/l] = Total creatine kinase [U/l] − (BB[U/l] + MM[U/l]).
○: M subunit of creatine kinase
□: B subunit of creatine kinase
AB_1: Antibody specific for M subunit
AB_2: Antibody specific for B subunit

Tab. 1. More frequently used immunologic assays for determination of enzymes and enzyme-inhibitor-complexes. Data on accuracy are not available as no international reference standards have yet been established.

Enzyme	Principle	Kit manufacturer[1])	Validation of method: Reference range or reference limit	Detection limit/ Sensitivity	Imprecision (CV%)[2]) N	A	Cross reactivity	References	Remarks[3])
Lactate dehydrogenase EC 1.1.1.27 isoenzyme 1	IP	1	<43 U/l (30 °C) 90–200 U/l (37 °C)	– –	2.6/4.7 3/3–5	–/– 4/8–15	None <0.1%	61 62–64	Double AB technique. IP with anti-subunit M-AB. Assay of supernatant LDH 1.
Aspartate aminotransferase EC 2.6.1.1 mitochondrial	IP	2	$\bar{x}$ = 0.5 U/l (37 °C)	–	5.7/7.4	0.7/1.5	–	65–68	IP with anti-cytosolic isoenzyme-AB. Assay of supernatant mitochondrial isoenzyme.
Creatine kinase EC 2.7.3.2 isoenzyme MB	II	3, 4	<10 U/l (25 °C) <24 U/l (37 °C)	3 U/l 0.001 ΔA/min	2/3 8/10	6/8 –/4.5	Specific for M subunit	22, 24, 25	II with anti-CK-M-AB. Assay of residual CK activity.
		5	<10 U/l (25 °C)	0.3 U/l	6.9/–	<5/<5	Specific for M subunit	19–20	Bioluminescence
	II+IP	1	<5 U/l (29.9 °C) <10 U/l (37 °C)	–	–/–	2–7/7	None	69–71	Double AB
	RIA	6	<25 µg/l	1.5 µg/l	7.8/15	3.1/5.1	100% CK-BB	72	Double AB
		7	< 7 µg/l	5 µg/l	9/2	13/7	100% CK-BB		Double AB
	IRMA	1	<30 µg/l	–	3/12	–/5	CK-BB	56, 73	Three AB.
		8	< 3.0 EU/l	1 EU/l	<10/<10	<10/<10	None	53	Solid phase anti-CK-B-AB, 2nd AB = labelled anti-CK-M-AB.
	ELISA	9	<10 µg/l	5.5 µg/l	18/–	6.5/–	None	57–59	Monoclonal AB. Solid phase anti-CK-M-AB, 2nd AB = labelled anti-CK-B-AB.
		10a	<10 µg/l	1.2 µg/l	4–14/8.1	3/3–9	None	54	Solid phase anti-CK-B-AB, 2nd AB = labelled anti-CK-M-AB.
Creatine kinase EC 2.7.3.2 isoenzyme BB	RIA	6	–	1 µg/l	4.5/3.7	2.2/3.3	41% CK-MB		Double AB
		8	<14 EU/l	2 EU/l	<10/<10	<10/<10	Specific for CK-B subunit		Double AB
		11	<12.5 µg/l	5 µg/l	11.2/9.4	–/7.5	Specific for CK-B subunit		Double AB
Lipase EC 3.1.1.3 pancreatic	RIA	12	30.2 ± 6.2 µg/l	–	–/–	2.8/7–14	–	74	
	ELISA	10	7.7–56 µg/l	0.3 µg/l	5.3/9.5	2.9/4.4	"Very low"	75	

Tab. 1. Continued.

Acid phosphatase EC 3.1.3.2 prostate	RIA	6, 11–19	2.0–7.2 µg/l[4])	0.2–1.0 µg/l	2–7/4–21	1–6/2–12	"None" to "Not completely specific"	76–79	Double AB
	IRMA	9	<2.0 µg/l	–	–/13	–/12	None	79	Monoclonal AB
	IAA	3	<1.0 µg/l	0.05 µg/l	2.9/3.0	1.9/0.9	None	80, 81	
		21	<0.65 U/l (37 °C)	0.04 U/l	3–4/13	3–4/5	–	35	
	ELISA	10	1.5–2.0 µg/l[4])	0.1 µg/l	4.5/7.5	5.5/8.7	None	83–85	
		19	<3.2 µg/l	0.5 µg/l	–/–	2.4/3.4	None	82	
		22	<2.0 µg/l	0.2 µg/l	8.7/12	6.9/8.6	None	86	Monoclonal AB
	FIA	15	<1.15 µg/l	0.5 µg/l	3.7/9.6	–/–	<0.5%		Double AB
Trypsin EC 3.4.21.4	RIA	10	140–400 µg/l	80 µg/l	7.5/5.2	2.7/7.3	–	87–90	
Elastase EC 3.4.21.37 Elastase-α1-proteinase-inhibitor complex	ELISA	3	20–180 µg/l	51 µg/l	8/8	4/3–7	None	91–92	

[1]) Kit manufacturers
1: Roche Diagnostics, Nutley NJ, USA.
2: Eiken Co. Ltd., Tokyo, Japan.
3: E. Merck, Darmstadt.
4: Boehringer Mannheim.
5: LKB Produkter, Bromma, Schweden.
6: Mallinckrodt Inc., St. Louis MO, USA.
7: Nuclear Med. Labs., Irving TX, USA.
8: International Immunoassays Lab., Santa Clara CA, USA.
9: Hybritech Inc., San Diego CA, USA.
10: Behringwerke Marburg.
10a: Behring Diagnostics, La Jolla CA, USA.
11: Nuclear Medical Systems, Newport Beach CA, USA.
12: Nuclin Diagnostics Inc., North Brook IL, USA.
13: Beckman Instruments, Brea CA, USA.
14: Becton Dickinson, Salt Lake City UT, USA.
15: BioGenex Laboratories, Dublin CA, USA.
16: Clinical Assays, Cambridge MA, USA.
17: Serono Labs. Inc., Braintree MA, USA.
18: Yang Labs Inc., Bellevue WA, USA.
19: New England Immunology Assoc., Cambridge MA, USA.
20: Leeco Diagnostics, Southfield MI, USA.
21: General Diagnostics, Morris Plains NJ, USA.
22: Abbott Lab., Chikago IL, USA.

[2]) Imprecision: N: "normal" resp. low concentration range: within run/day to day. A: "abnormal" range: within run/day to day.

[3]) Abbreviations:
AB: antibodies
ELISA: enzyme-linked immunosorbent assay
FIA: fluorescent immuno assay
IAA: immuno adsorption assay
II: immuno inhibition
IP: immuno precipitation technique
IRMA: immuno radiometric assay

[4]) Upper reference limits.

antibodies inhibits all creatine kinase M subunits in a serum sample. The activity of the B subunit of creatine kinase-MB remains virtually uninfluenced. As the isoenzyme BB only very rarely occurs in blood in spectrophotometrically measurable activity (i.e. >3 U/l), this test enables the isoenzymes MM and MB to be differentiated quickly and easily. After total inhibition of creatine kinase-MM, creatine kinase-MB can be detected by means of its still active B subunit without a separation stage (e.g. centrifugation) (22). Monoclonal antibodies are also able to inhibit the activity of the creatine kinase M subunit selectively and totally, however they appear to possess no definite advantages over polyclonal antibodies in immunoinhibition tests (23).

In the determination of creatine kinase-MB in the immunoinhibition test, interferences, i.e. false positive results, are caused by macromolecular creatine kinases as is also the case with immunoprecipitation and furthermore by increased creatine kinase-BB activities (21). In spite of this disadvantage the immunoinhibition test was proved especially successful in cardiac infarction diagnosis due to its rapidity and easy application (24, 25).

Catalytically inactive enzyme molecules are not detectable with the immunoprecipitation and inhibition procedures; however if they occur in a high concentration and cross react with the antiserum, they may cause interferences in the immune reaction of active enzymes. As is shown in figure 3, such cross reactions, e.g. between inactive creatine kinase-MM and inhibiting anti-creatine kinase-M antibodies, can be verified in the displacement experiment and their order of magnitude estimated.

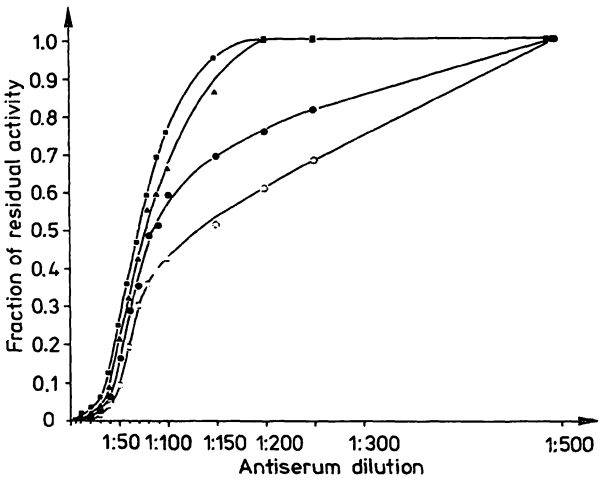

Fig. 3. Displacement of active creatine kinase-MM from a specific anti-creatine kinase-M antibody by inactive creatine kinase-MM. Creatine kinase-MM was inactivated at 37 °C in a pH-controlled serum matrix (pH 7.4 ± 0.1). Varying concentrations of inactivated creatine kinase-MM were mixed with a stepwise diluted antiserum containing inhibiting anti-creatine kinase-M antibodies. The mixtures were pre-incubated for 20 h at 4 °C followed by addition of catalytically active creatine kinase-MM (520 U/l). After a second incubation for 3 h at room temperature enzyme catalytic activities were measured in triplicates.
Y-axis: Ratio of residual to initial catalytic activity.
Molar ratio of inactive to active creatine kinase-MM:
○–○–○: 0.0 (active enzyme only)
●–●–●: 4.8
▲–▲–▲: 12.1
■–■–■: 24.2

Immunoadsorption

The immunoadsorption assay comprises three reaction stages: the binding of the desired enzyme by specific antibodies, the subsequent separation of the resulting enzyme-antibody-complexes and the final quantification of the complex associated enzyme activity. Only those antibodies are suitable which do not inhibit (26, 27) or only slightly inhibit (28, 29) the enzyme catalytic activity. Antibodies which lead to a stabilization (30, 31) and/or reactivation or activation (32, 33) of the enzyme are especially desirable for the detection of catalytically unstable enzymes such as acid phosphatase. The separation of the enzyme-antibody-complexes is carried out by means of precipitation with ammonium sulphate (34) or polyethylene glycol (35). After centrifugation and washing it is possible to resuspend the enzyme complexes and to measure their activity. Solid-phase assays in which the enzyme is bound to immobilized antibodies are methodically more refined (fig. 4).

The immunoadsorption test detects an enzyme *directly* and thus offers some considerable advantages over the precipitation and inhibition methods. In addition its sensitivity can be increased by augmenting the volume of the test sample: the enzyme activity is enriched by the immunoadsorbent before it is measured (29). The obligatory separation step offers additional advantages. It eliminates all potential interference factors in the test matrix before the actual enzyme determination and thus improves the specificity of the assay.

Immunoadsorption assays are distinguished by a wide measuring range (28, 38, 39) and a remarkably high analytical sensitivity. As is shown in the prostate acid phosphatase tests, they are able to measure catalytic concentrations of less than 0.1 U/l. This corresponds to the enzyme mass concentration also considered to be the lowest detection limit for radioimmunological and immunometric procedures (tab. 1). As the immunoadsorption test is always carried out with an antibody excess, interferences can be caused by unspecific antibodies if polyclonal an-

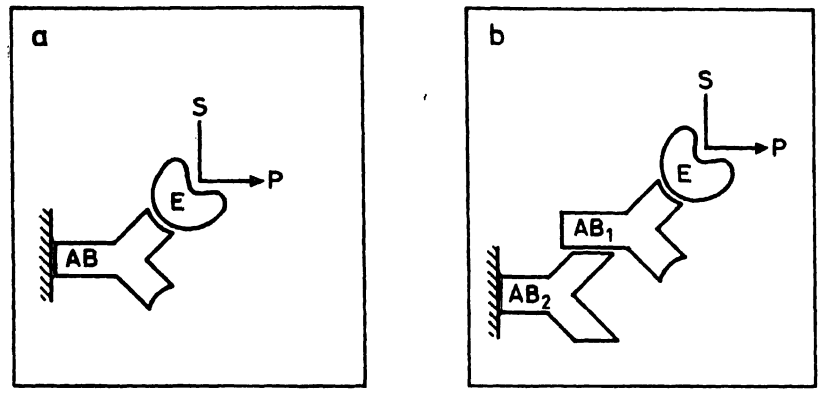

Fig. 4. Solid-phase immunoadsorbent assays for prostatic acid phosphatase (a) or placental alkaline phosphatase (b) (36, 37). A polystyrene-attached polyclonal antibody (AB, AB_2) reacts directly or mediated by a monoclonal antibody (AB_1) with the appropriate enzyme (E). The amount of immunoreactive phosphatase is determined by its catalytic activity in the immune complex.
S: substrate
P: product of catalytic activity

tiserum is used. This principal disadvantage can however be eliminated by the introduction of monoclonal antibodies (fig. 4b).

Radioimmunoassay

Problems associated with the determination of the concentration of enzymes can be seen from the description of radioimmunoassays used to detect creatine kinase MB (tab. 1).

It is not possible to obtain isoenzyme specific anti-creatine kinase antisera by traditional immunization, but only those which are specifically directed against B or M subunits of creatine kinase. For this reason only creatine kinase-B RIAs (assaying BB *and* MB) or creatine kinase-M RIAs (assaying MM *and* MB) are available (24, 40–44). The possibilities of interference in a radioimmunological creatine kinase-MB determination in human serum are thus already predetermined: the creatine kinase-M RIA is generally unsuitable due to its cross reaction with the creatine kinase-MM which occurs in a concentration about 10 times greater; the creatine kinase-B RIA (fig. 5) will only be unaffected by physiological creatine kinase-BB concentrations if the creatine kinase-MB concentration to be measured is considerably higher than BB, i.e. if MB is clearly within the pathological range. Due to this insufficient specificity a creatine kinase-B RIA, in spite of its greater sensitivity, is not able to detect the increase of creatine kinase-MB in the serum of an infarction patient earlier than the immunoinhibition test.

Another decisive disadvantage of the radioimmunoassay is the long time it requires. Especially in the diagnostics, e.g. of an acute cardiac infarction, it will

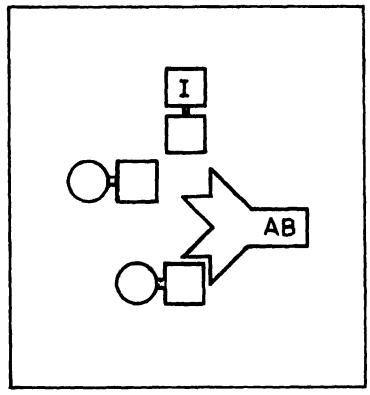

Fig. 5. Detection of creatine kinase-MB by radioimmunoassay. Labelled creatine kinase-BB and unlabelled creatine kinase-MB compete for the binding sites of a limited amount of an anti-creatine kinase-B antibody (AB).
○: M subunit of creatine kinase
□: B subunit of creatine kinase
I: ^{125}I-labelled B subunit

hardly reflect the current clinical condition of the patient. The analytical sensitivity of most creatine kinase-B RIAs lies in the range 1 to 5 µg/l (tab. 1). For active creatine kinase-BB or -MB with a specific activity of ≥400 U/mg protein (45), radioimmunoassays of this kind have a lower detection limit of ≥0.4 U/l. If a lower specific activity (e.g. 10 U/mg) is taken as the basis, a test sensitivity which is too high will inevitably be simulated (46). Thus, although these RIAs are 10 times as sensitive as the immunoinhibition, they are by no means more sensitive than luminometric creatine kinase-B assays (18, 19, 20).

An interesting variant is a radioimmunoassay which works with creatine kinase-BB binding autoantibodies, isolated from patients' serum containing these autoantibodies (47, 48). These highly affine antibo-

dies (association constants: 1.4×10^{11} l/mol) show no cross reactions with the isoenzymes MB and MM under test conditions even when they are present in a 10^4 or 10^5 times greater concentration than creatine kinase-BB. This unparalleled isoenzyme specific creatine kinase RIA is remarkably sensitive (detection limit: 1 µg/l creatine kinase-BB), but can only detect catalytically active enzyme.

The detection of inactive enzyme forms would be particularly important in the case of an unstable enzyme such as creatine kinase BB, which has moreover been assigned a tumour associated marker (49). Although cross reactions have been observed with products of in vivo and in vitro enzyme degradation in some creatine kinase-B RIAs, up to the present all pinpointed attempts to quantify inactive creatine kinase-B protein in human serum with an immunoassay have failed (40, 43, 50). Here, the excessively high concentrations (15—198 mg/l (!)) of inactive creatine kinase-B protein measured in the serum of healthy persons appear to be very doubtful (51, 52).

Immunometric assay

For the immunometric procedure, two antisera are needed. Their antibodies must be capable of reacting with the different respective epitopes of the enzyme to be detected without impeding each other sterically in the process. The antibodies of one of the antisera are immobilized and separate the enzyme in the test sample. The antibodies of the second antiserum are labelled (radio iodine (IRMA), marker enzyme (ELISA)) and identify the enzyme.

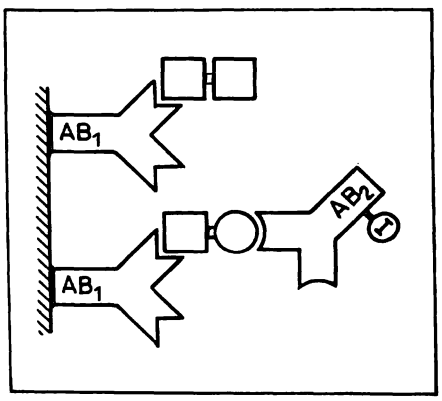

Fig. 6: Immunometric assay for creatine kinase-MB. In the 1st step immobilized anti-creatine kinase-B antibodies (AB_1) only bind the isoenzymes BB and MB. In the 2nd step the isoenzyme MB is then specifically detected by a ^{125}I-labelled (53), respectively peroxidase conjugated (54) anti-creatine kinase-M antibody (AB_2).
I: label

In figure 6 an immunometric creatine kinase-MB assay is represented. In a first reaction phase the isoenzymes MB and BB are bound to immobilized creatine kinase-B antibodies. After a separation or washing step they are separated from the remaining creatine kinases in the serum (MM, macromolecular creatine kinases). In the second reaction phase the differentiation of both the antibody associated isoenzymes occurs: only creatine kinase-MB can bind labelled creatine kinase-M antibodies.

Such an assay can be very specific and the creatine kinase-MB in a serum sample can be measured without interference even in the case of a 1000-fold molar excess of the isoenzymes BB and MM (55). However, with a detection limit of 10 µg/l of enzyme protein, the assay does not match the sensitivity of the radioimmunoassay (tab. 1), even though it is just as time and labour consuming. By shortening the reaction times and dispensing with the separation stage between both reaction phases, the immunometric assay is made more acceptable for clinical requirements, but at the same time, its analytical specificity and sensitivity are reduced (56). The extent to which the introduction of monoclonal creatine kinase-B and creatine kinase-M antibodies (57—59) is able to overcome the weaknesses of the immunometric assay has yet to be clarified through further investigation.

Perspectives

The trend in clinical enzyme diagnostics away from the "indirect" assay (e.g. immunoprecipitation) towards the "direct" assay (e.g. immunoadsorption) will continue and lead to a further improvement in the specificity and sensitivity of immunological enzyme assays. For the same reasons the non-competitive immunometric methods will replace the classical competitive immunoassay.

The use of monoclonal antibodies should play a decisive role in both these developments. Monoclonal antibodies represent a defined homogeneous reagent in terms of epitope, affinity and reactivity, which is available in virtually unlimited quantities. Thus new perspectives are being opened up for the standardization and application (e.g. differentiation of post-synthetically altered and inactive enzyme forms) of immunological enzyme tests. In the case of acute illness, such as an acute myocardial infarction, the diagnosis has to be carried out under emergency conditions. In addition to a quick and practicable creatine kinase-MB test with limited specificity (immunoinhibition), highly specific, but more time and

labour consuming assays (tab. 1) are at present available. It remains to be seen whether this dilemma can be resolved by an acceleration of immune reactions by means of sonication (acceleration factor >100(!)), as has been demonstrated just recently (60).

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Immunohistochemical Procedures: Applications and Problems[1])

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Summary: The introduction of immunohistochemical methods into pathological diagnostics has opened new possibilities for combining the specificity of immunological methods with the selectivity of morphological techniques. This considerable expansion of diagnostic possibilities has occurred since the introduction of monoclonal antibodies. The basic conditions of immunohistochemical reactions (fixation, embedding), fundamental differences between the use of monoclonal antibodies and that of polyclonal antisera and possibilities for increasing the sensitivity of diagnoses are discussed and illustrated by practical examples.

Immunhistochemische Methoden: Ihre Anwendung und Probleme

Zusammenfassung: Die Einführung immunhistochemischer Methoden in die diagnostische Pathologie hat neue Möglichkeiten für die Kombination der Spezifität immunologischer Methoden mit der Selektivität morphologischer Techniken eröffnet. Seit der Einführung monoklonaler Antikörper haben die diagnostischen Möglichkeiten wesentlich zugenommen. Die Grundvoraussetzungen für die immunhistochemischen Reaktionen (Fixierung, Einbettung), grundsätzliche Unterschiede zwischen der Anwendung von monoklonalen Antikörpern und der von Polyseren sowie die Möglichkeiten der Verbesserung der Empfindlichkeit diagnostischer Verfahren werden diskutiert und anhand praktischer Beispiele dargestellt.

Introduction

Pathological-anatomical diagnosis is, to a certain extent at least, subjective. It is based on the evaluation of more or less selectively stained paraffin or frozen sections, or, in the case of cytological diagnoses, cell suspensions or smears. The reliability of the diagnosis depends above all on the experience of the investigator. The diagnostic difficulties increase even for the experienced pathologist as the morphological picture becomes less characteristic or more "foreign". Nevertheless, the opinions and conclusions from analogy of the morphological observer often form the basis from which therapeutical consequences of great weight are drawn. The methodology of morphological diagnostics depends on the ability to recognize characteristic forms (1), and for a long time such diagnoses could not be verified by more specific, functionally oriented techniques.

In the last few years immunohistochemistry has brought about a revolution. It allows the investigator to combine the remarkable specificity of immunological reactions with the extraordinary topographical resolution of light and electron microscopy and thus to gain basic information on the cellular and tissular composition of objects under investigation. The additional information obtained in this way could not be obtained with biochemical, immunochemical or morphological techniques alone.

Since *Coons* (2, 3) first succeeded in visualizing tissue constituents histologically by means of immunofluorescence techniques in 1941, the popularity of this technique has increased exponentially. Since the preparation of monoclonal antibodies (4), this increase may even be deemed explosive.

In the following we wish to present the immunohistochemical techniques that are in pathological-ana-

[1]) Presented at the Kleinkonferenz „Immunologische Diagnostik" der Deutschen Gesellschaft für Klinische Chemie, Hamburg, Juni 1983.

tomical usage and to discuss their reliability and informative value. The choice of procedures presented here is based on our own experience.

Preparation of tissues for immunological investigations

In the following we will restrict the presentation of the technical procedures involved in immunohistochemical investigations to the processing of formalin-fixed, paraffin-embedded tissue and cryostat sections of fresh tissue. Paraffin-embedded material has the great advantage that it is possible to perform retrospective evaluations of a large pathological shipment. If one is limited to cryostat sections, only prospective investigations can be performed, and there are considerable problems involved in obtaining, shipping and storing tissues.

Fixation

For all research-oriented problems and all known antigens, the best, most exact and most reliable results are obtained in cryostat sections after brief fixation in organic solvents, such as acetone. How well the structure is preserved depends on the length of fixation time and on the avoidance of artefacts due to freezing. The shock freezing technique via freon or arcton as interphase with liquid nitrogen has proved in practice to be the method that does the least damage to the tissue. The frozen sections that are prepared in this manner can be stored at −80 °C and are fixed with acetone prior to immunohistochemical staining. This procedure is adequate for most antigens but is not applicable when immunohistochemical procedures are planned for electron microscopy ((5), fig. 1). In this case short-term fixation with various fixatives, such as glutaraldehyde, paraformaldehyde and diluted *Karnovsky*'s fixation medium, is applied. This guarantees on the one hand that the structure is preserved quite well, and on the other hand many of the membrane antigens investigated so far can be demonstrated. One must, however, expect a loss in sensitivity that varies from antigen to antigen.

Embedding

Until only a few years ago immunohistochemical techniques were held to be unfeasible and unreliable for routine paraffin-embedded material. The demonstration of immunoglobulins on routinely embedded formalin-fixed material (6) was a decisive turning point for immunohistochemical diagnostics. In subsequent years advances were made in diverse

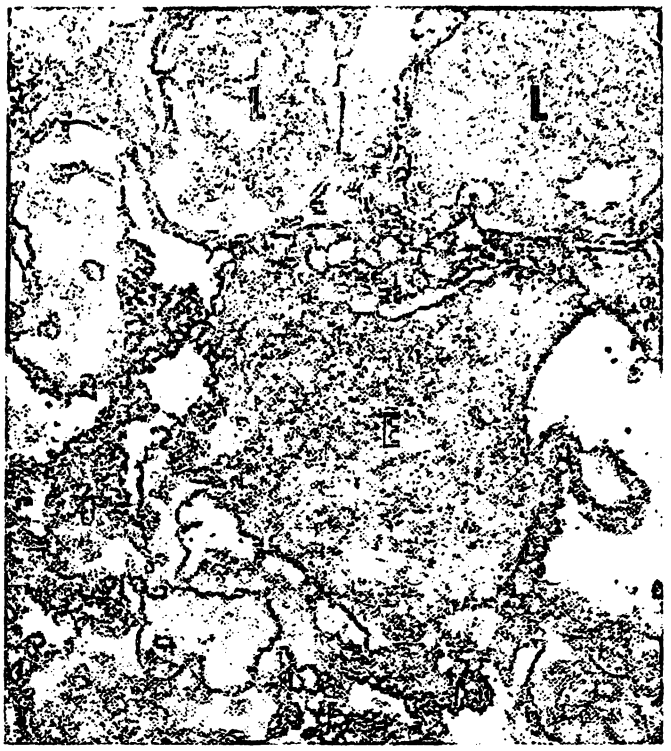

Fig. 1. Immuno-electron microscopic localization of HLA-DR on an epithelial cell (E) of the thymic cortex. The immune reaction is restricted to the outer cell membrane. Lymphocyte (L). 5700 ×.

Tab. 1. Comparison of demonstration of commonly used antigens in paraffin-embedded tissue and in cryostat sections.

	Paraffin Post-embedding	Cryostat
Cell membrane		
Membrane-antigens and receptors	−	+
Lectin receptors	+	+
Cytoplasm		
Secretion products		
Hormones, mucus	+ −	+
Immunoglobulins	+	+
Intermediary filaments		
Actin, myosin, vimentin, desmin etc.	− +	+
Nuclear antigens		
e.g. Tdt (Terminal deoxynucleotidyl-transferase)	−	+
ANF (Anti nuclear factors)	+	+
Tumour associated antigens		
CEA (Carcinoembryonic antigen)	+	+
α-Foetoprotein	+	+
etc.		

areas, particularly with intracellular antigens, by increasing the sensitivity of the techniques (tab. 1). With adequate embedding, many antigen structures can be demonstrated in embedded material. Better preservation of the structure and increasing thinness of the histological sections also improve the results. It is generally recognized that particularly intracytoplasmic antigens with a relatively high antigen density can be easily demonstrated in paraffin-embedded material, provided the fixation is appropriate (fig. 2). Examples of this are primarily immunoglobulins (7, 8), hormones, intracytoplasmic filaments and, under appropriate conditions, certain intranuclear antigens (9, 10). In contrast, the demonstration of membrane antigens is fraught with difficulties ((11), tab. 1). It has not yet been possible to demonstrate the large number of membrane antigens defined by monoclonal antibodies reliably in paraffin-embedded material. An exception to this are lectin receptors of the cell membrane, i.e., sugar structures, which are not altered by fixation and can thus be recognized optimally in paraffin sections (fig. 3).

The dream of the morphologist, to be able to combine the specificity of immunohistochemical methods with the optimum preservation of morphological structure in plastic-embedded material, has only been approximated so far. Immunoglobulins (fig. 4) and hormones, in particular, can be demonstrated in plastic-embedded material, under certain conditions (12).

In practice immunohistological techniques have been of particular significance for us for the identification and specification of malignant lymphomas. We have taken advantage of the difference in sensitivity between paraffin-embedded material and frozen sections, in order to distinguish between secretory immunoglobulins and membrane globulins (13).

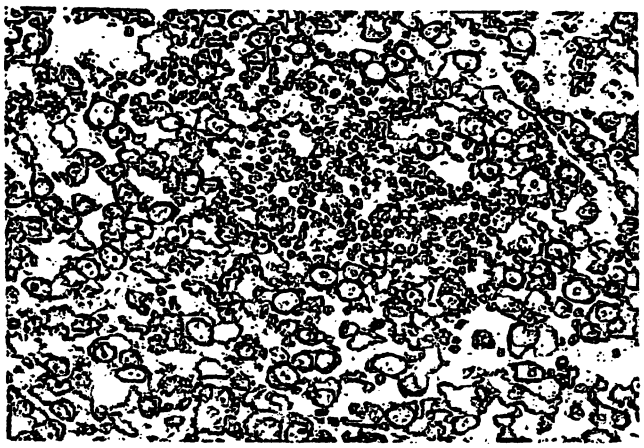

Fig. 2. Non-*Hodgkin*'s lymphoma of high grade malignity (immunoblastic lymphoma). Demonstration of the light immunoglobulin chain kappa in a paraffin section using the peroxidase-antiperoxidase (PAP) method according to *Sternberger*. The tumour cells show a positive intracytoplasmic immune reaction. 370 ×.

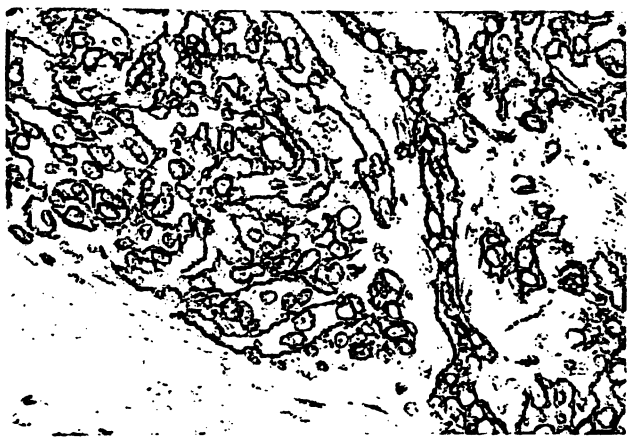

Fig. 3. Benign haemangioendothelioma. The endothelial cells of the vessels show a positive reaction with *Ulex europaeus* lectin (photo Dr. *B. Borisch*). 370 ×.

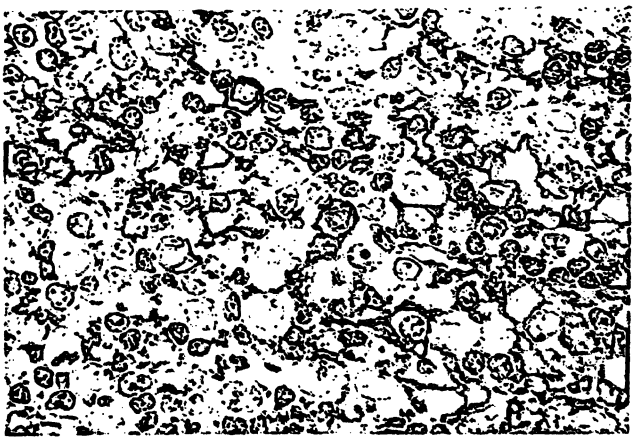

Fig. 4. Demonstration of the light immunoglobulin chain lambda in blast and plasma cells of the tonsil on semithin sections by means of the peroxidase-antiperoxidase (PAP) method. 370 ×.

Antibodies

The quality and specificity of immunological demonstrations depend fundamentally on the type and quality of antibodies used. The scientific and practical consequences of the breakthrough achieved with the introduction of monoclonal antibodies (4) cannot be appraised yet. There is no doubt that the introduction of these highly specific and pure reagents has initiated a revolution in the field of pathological-histological and cytological diagnoses. Previously the naming – recognition and description of the cells of a tissue were reserved for a few, particularly experienced specialists. Now the immunochemical demonstration of cell-specific, under certain conditions,

functionally specific, molecules has created possibilites for bridging the gap between descriptive anatomy and pathology, and the function (fig. 5). Let us briefly discuss a few basic considerations on the antigenic structure of cell surfaces (14).

Fig. 5. Thymus of a 4 year old boy. T lymphocytes show a positive immune reaction with the monoclonal antibody Leu-1. Mature medullar thymocytes react much more strongly than cortical thymocytes do. Some of the latter are negative. In the center of the picture a *Hassall*'s body. Frozen section. 250 ×.

Antigenic Structure of Cell Surfaces

When different animal species are immunized, different antigenic structures are recognized on cell surfaces, depending on which determinants of the cell surface are recognized by the immunized animal species as foreign. If different animal species, e.g., rabbit, sheep, horse and monkey are used for the production for antihuman lymphocyte sera, the different spectra of the antisera reveal a very variable pattern of reactivity with human lymphocytes and bone marrow cells, in spite of the similarity of the immunogen and a comparable cytotoxic antilymphocyte titre (15). Since thus far the only animal species available for immunization for the production of monoclonal antibodies are rat and mouse, it is quite conceivable that particularly interesting antigenic structures and determinants in certain experimental systems, that have already been defined with the aid of polyclonal antibodies, are not yet demonstrable with monoclonal antibodies.

Monoclonal antibodies have found particular application for the recognition of cell type-specific determinants (16). We can assume that a particular degree of cell type specificity has been achieved whenever molecules that are known to be relevant to cell functions are identified by means of immunohistochemical procedures. When determinant specific monoclonal antibodies are used, however, we must expect to find considerable unexpected and functionally irrelevant cross-reactivity and partial antigen associations with other cells and structures. The reason for this may lie in what may be called nature's economy principle, in the sense that cellular individuality is not based on one single characteristic antigenic determinant, but on a pattern of different antigenic structures with variations in their distribution on the cells (17). This principle is familiar to all of us from the polymorphism of the HLA system or the *Kaufmann-White* scheme of *Salmonella*. Why should nature breach this tested principle when it comes to cell type specificity?

For the practical demonstration of so-called cell type-specific determinants by means of monoclonal antibodies, this means that it is exactly these cross-reactivities and partial antigen associatons that make the application of monoclonal antibodies problematic. Let us explain this briefly in the following diagram (fig. 6). A polyclonal antibody shows definite quantitative differences in its reactions with cells A, B, and C, due to the varying determinant specificity. These considerations make it clear that the methods used in conventional immunohistology, namely, dilution to increase specificity, are of no value when monoclonal antibodies are employed. On the contrary, it is important to recreate the antigen mosaic demonstrated here by combining the demonstration of different antigens via double labelling methods.

Immunohistochemical Techniques

Almost as mumerous as the available antibodies with their varying specificities are the immunohistochemical techniques that are employed; moreover, their specificity and sensitivity are being constantly increased at the same time as their application is being simplified (18). Fluorescence procedures, which

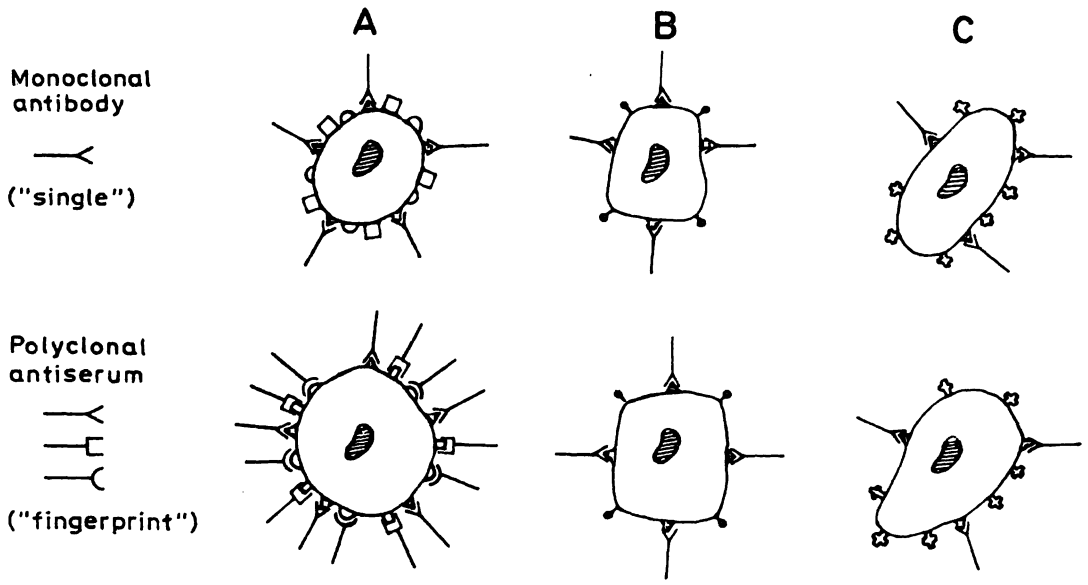

Fig. 6. Cell type specificity and cross-reactivity as a problem of immunological cell recognition. In this example of a determinant-specific reaction the monoclonal antibody is not able to distinguish between cells A, B, and C, although all 3 cells types have very different antigen patterns; this is clearly demonstrated by the polyclonal antiserum, which distinguishes the pattern of antigen associations on the cell membrane.
(Reproducted with permission of Prof. Dr. Dr. *W. Müller-Ruchholtz* (14)).

were the first to be introduced, are equal to the later peroxidase technique with regard to specificity and sensitivity, but have certain decisive disadvantages. For instance, specialized microscopy is needed; fresh tissues must be used; morphology is poor, and fluorescent stains are impermanent. For these reasons we employ almost exclusively peroxidase and, more recently, alkaline phosphatase immune labelling techniques. The evaluations can then be performed under a conventional light microscope, often on paraffin sections, and the permanence of the immunostaining is excellent. Out of the multitude of immunohistochemical techniques let us illustrate here three of the most important methods, which are probably also of the most practical significance (fig. 7). In the direct peroxidase technique the tissue is incubated with peroxidase-conjugated antibodies. The applied antibody combines with the antigen to be demonstrated and, after addition of diaminobenzidine as substrate, it is visualized in the form of a brown reaction product (19). Some disadvantages of this simple method are: For each antigen one needs a different peroxidase-conjugated antibody. Due to the conjugation reaction between antibody and peroxidase, some of the antibody denatures and is thus less specific for the antigen to be demonstrated. The binding of antibody and peroxidase is never complete, so that free excess antibody and free peroxidase can lead to nonspecific reactions. An increase in specificity, and thus a reduction of background staining is made possible by the indirect peroxidase method (20). In this method a primary antibody is applied followed by a secondary peroxidase-conjugated antibody that is directed against the globulin fraction of the primary antibody. Further progress was represented by the peroxidase technique according to *Sternberger* et al. (21), which made peroxidase-antibody conjugation superfluous. In this procedure the sections are incubated with the primary antibody and then with a secondary antibody directed against the globulin fraction of the primary antibody. This step is followed by the peroxidase-antiperoxidase complex, which must be prepared in the same animal species as the primary antibody. For the visualization of antigens on frozen sections, we find the indirect peroxidase technique to be particularly appropriate, and on paraffin sections, the peroxidase reaction ac-

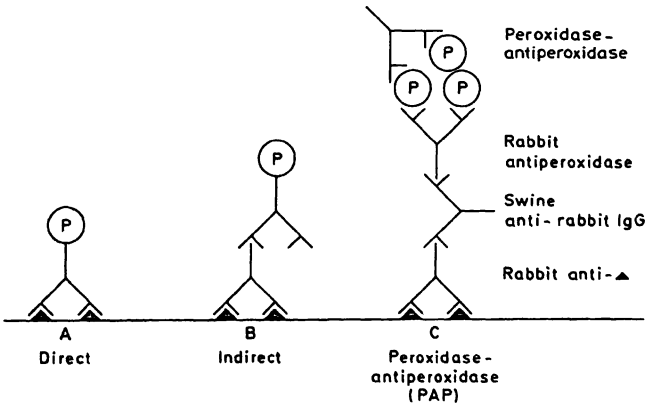

Fig. 7. Diagrammatic representation of antigen demonstration by means of the direct immunoperoxidase method, the indirect immunoperoxidase method and the peroxidase-antiperoxidase (PAP) method. ▲ antigen.

cording to *Sternberger*. Independent of the method employed, the specificity was increased by such means as

(1.) highly diluting the primary antibody and using long incubation periods,

(2.) absorbing the secondary antisera with human serum, and

(3.) proteolytic digestion of paraffin sections with proteases (22), to mention only a few.

A procedure used more and more often recently is the biotin-avidin system ((23), fig. 8). This is based on the principle that the glycoprotein avidin has a high affinity for biotin. Since avidin also has four binding sites for biotin, avidin-biotin-peroxidase complexes can be prepared that have the advantage that an antigen is demonstrated not with one, but with several peroxidase molecules, which leads to a significant increase in the strength of the reaction.

In addition to the possibility of visualizing a single antigen in the tissue, the desire soon arose to visualize two or more antigens simultaneously. This led to the development of a large number of double and multiple labelling techniques (24). For instance, one antigen is visualized with peroxidase, i.e., a brown reaction product, and a second antigen is visualized with alkaline phosphatase, which appears, e.g., red, depending on the substrate used, or with colloidal gold, which also produces a red stain. Meanwhile it is also possible to perform double labelling with monoclonal antibodies (25).

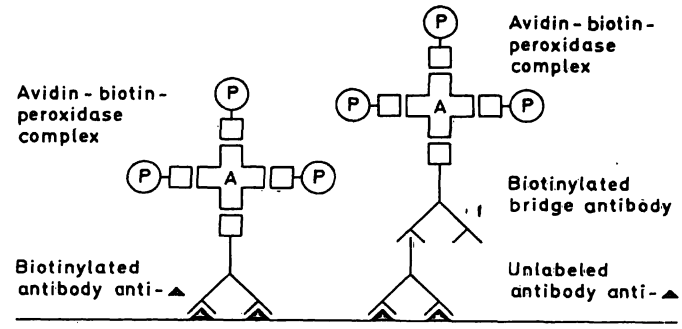

Fig. 8. Antigen demonstration by means of the biotin-avidin system. Direct and indirect demonstration. □ biotin, ▲ antigen, A-avidin (diagram according to *Falini & Taylor* (24), modified.

If we observe immunological techniques from the viewpoint of a clinical chemical test, we can formulate similar criteria of quality. The immunohistochemical procedures used today thus possess, as described in greater detail by *Petrusz* et al. (26), high precision, sensitivity and specificity.

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Diagnostic Concepts in Clinical Organ Transplantation: Histocompatibility Testing and T Cell Monitoring[1])

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Summary: Immunological rejection is still the most important problem in clinical organ transplantation requiring the lifelong immuno-suppressive treatment of the allograft recipient. The clinical success rate however can be improved by a variety of diagnostic procedures:

a) The reduction of the histocompatibility barrier between donor and recipient by HLA-typing and organ exchange in order to obtain the best possible match;

b) posttransplant monitoring of donor specific immune responsiveness towards donor antigens;

c) monitoring of T-cell subsets by monoclonal antibodies and cytofluometry.

T-cell subset monitoring is a rapidly developing new technique with great potential. With the presently available first generation reagents already a detailed analysis of the major T-cell population in circulation is possible, which can markedly facilitate the diagnosis of rejection and viral infection. New monoclonal antibodies, defining certain activation stages of T-cells or permitting a more refined differentiation of functionally distinct subsets will lead to increased relevance of this diagnostic approach. A particulary interesting aspect of monoclonal antibodies against T-cell differentiation antigens is their potential therapeutic use as immunosuppressive drugs with a highly selective action on defined T-cell subsets.

Immunologische Diagnostik bei Organtransplantationen: Histokompatibilitätstestung und T-Zell-Monitoring

Zusammenfassung: Die immunologische Abstoßung ist das zentrale Problem der klinischen Organtransplantation. Neben der immunsuppressiven Therapie spielt die immunologische Diagnostik für die Herabsetzung des Abstoßungsrisikos und die Früherkennung von Abstoßungsreaktionen und Infektionen eine wichtige Rolle. Klinisch bedeutsame diagnostische Konzepte sind:

a) Die Reduktion der Histokompatibilitätsdifferenz zwischen Spender und Empfänger durch prospektive Gewebetypisierung in Kombination mit überregionalem Organaustausch zur Erzielung einer möglichst guten Gewebeübereinstimmung;

b) Untersuchungen der zellulären Immunaktivität gegen die inkompatiblen Antigene des Transplantates im Verlauf nach der Transplantation;

c) die Überwachung der Konzentration der unterschiedlichen T-Zell-Subsets im Blut mit Hilfe von monoklonalen Antikörpern und Zytofluometrie.

Die Analyse von T-Zell-Subsets anhand von Differenzierungsmarkern, die durch monoklonale Antikörper definiert sind, stellt eine sich schnell entwickelnde neue Technik dar, die im Bereich der Organtransplantation zunehmend an Bedeutung gewinnt. Mit der jetzt verfügbaren ersten Generation von Antikörpern ist bereits

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eine gute Differenzierung zwischen Zellen mit Helferfunktion und zytotoxischen/supprimierenden T-Zellen möglich. Die Bestimmung des relativen Anteils dieser T-Zell-Subsets im Blut kann die Differentialdiagnose zwischen Abstoßung und Virusinfektion wesentlich erleichtern. Das zunehmende Verfügbarwerden von monoklonalen Antikörpern, die bestimmte Aktivierungsstadien von Lymphocyten erfassen oder zu einer Verfeinerung der Subset-Differenzierung führen, dürfte diesen diagnostischen Ansatz schon bald weiter an Bedeutung gewinnen lassen. Ein besonders interessanter Aspekt der monoklonalen Antikörper gegen T-Zell-Differenzierungsantigene ist ihr therapeutischer Einsatz als Immunsuppressiva mit hochselektiver Wirkung gegen definierte Lymphocytenuntergruppen.

Introduction

Organ transplantation is a field of increasing clinical relevance. Transplantation of the kidney has already become a routine procedure for the treatment of chronic renal failure and the transplantation of other life sustaining organs such as liver, heart and pancreas has also left the experimental stage and is currently rapidly expanding. The most important problem of this type of treatment however has remained immunological rejection of the graft. It reflects the response of the recipient's immune system towards the incompatible alloantigens of the graft. Empirically developed immunosuppressive drug regimens permit the effective control of the rejection process in most but not all patients.

Clinically the rejection process manifests as a deterioration in graft function, finally leading to graft failure. In the hyperacute and acute types of rejection this develops rapidly, in chronic rejection more slowly over a period of months or even years. The most important diagnostic procedures for detecting rejection are graft biopsy and follow up studies of graft function. From the immunologists point of view, this is unsatisfactory because it should be possible to detect the rejection response on the level of the immune system before graft damage has occurred. Furthermore the testing of the transplantation antigens of recipient and donor should even help to prevent the occurrence of untreatable rejection processes. It is the aim of this paper to review and evaluate the current state of these diagnostic concepts in organ transplantation.

Histocompatibility Testing

Histocompatibility or transplantation antigens are all surface molecules which are able to induce allograft rejection after transplantation in a recipient not possessing the same antigens. The aim of tissue typing (histocompatibility testing) is the detection of these antigens in donor and recipient and the subsequent selection of the combination with the best match, ideally full compatibility between donor and recipient. Tissue typing has provided the basis for understanding the immunogenetics of transplantation and is an established clinical procedure. The actual effect on the clinical outcome of organ transplants however has been limited and depends on a number of additional factors.

In clinical practice tissue typing means typing for HLA antigens. The HLA system represents the major histocompatibility system (MHC), which is controlled by a cluster of genes located on the short arm of the sixth chromosome (1, 2). It codes for two classes of cell surface proteins. HLA loci A, B and C code for class I antigens consisting of a polymorphic polypeptide chain of 44000 Dalton and an invariant chain of 12000 Dalton representing cell surface bound β_2-microglobulin. Class II antigens are controlled by multiple loci in the D/DR, DC and SB regions on the HLA chromosomal segment and consist of two polypeptide chains with molecular weights of 28000 and 34000. Whereas class I antigens are present on all or nearly all cells, the expression of class II antigens is more restricted (B cells, macrophages and a few more cell types). The outstanding feature of the HLA system is the enormous allelic variation of its gene products (genetic polymorphism). There are more than 20 well defined A locus alleles and more than 40 B locus alleles. This polymorphism is the cause of the tremendous variability of individual HLA patterns (1, 2).

HLA antigens are called strong transplantation antigens because they play a dominant role in the induction of rejection responses. It has recently become obvious that this strong effect of the HLA antigens in transplantation is a result of their role in antigen recognition by T cells in general. It is now clear that T lymphocytes can recognise antigen only when it is presented in the context of HLA antigens (3). This restriction of the antigen recognition process by MHC antigens is one of the mechanisms by which HLA antigens are involved in the regulation of immune responses (Ir gene effect) and probably also

the cause for the association of certain diseases with particular HLA antigens (4, 5). These aspects of the HLA system have raised a general interest in the system going far beyond the transplant problem, but have also contributed to a better insight into its particular role in organ transplantation.

The technical problems of HLA typing can be regarded as essentially solved. The antigens coded for by the three class I loci and the DR and DC class II antigens are typed for by complement-dependent cytotoxicity with lymphocytes as target cells and defined monospecific typing sera (2). The antisera are obtained from immunized human individuals (multiparous women, transfused patients and transplant recipients). By this means in many instances complete antigen patterns can be obtained. Besides HLA typing, histocompatibility testing includes typing for ABO blood group antigens and screening for the presence of cytotoxic antibodies (tab. 1). Before the transplantation is actually performed a cross-match with recipient serum and donor lymphocytes is necessary. In the presence of a positive cross-match with a recent serum of the patient the transplantation should not be performed because hyperacute rejection is impending (2).

Despite the advanced state of HLA typing the influence of tissue matching on transplant results is limited. Nevertheless it is worthwhile to consider what HLA typing can contribute and what the reasons for its limited impact are. Clear evidence for the dominant role of the HLA system in organ transplantation has been derived from kidney transplants performed in living related combinations particularly in siblings. If siblings share all HLA antigens (two haplotype identity) the organ graft survival is about 90%, if there is identity for antigens coded for by one chromosome (one haplotype identity) this is 75—80% and if there is a two haplotype mismatch it is the same as in unrelated combinations (6, 7).

Tab. 1. Tissue typing for organ transplantation.

1. Recipient	
a) Typing for ABO	at the time of entry in the waiting list
b) Typing for HLA	
c) Screening for HLA antibodies (indicating presensitization)	
2. Donor	
a) Typing for ABO	
b) Typing for HLA	
3. Cross-match (complement-dependent cytotoxic assay with recipient serum and donor lymphocytes)	

For a long time it has been difficult to prove the beneficial effect of matching in unrelated donor recipient combinations. Important reasons for this were the incomplete knowledge of the HLA system and its enormous polymorphism, rendering it very difficult to find combinations with full identity. Only by an extensive organ exchange between transplant centers has it become possible to achieve a sufficient number of good matches and to collect enough data to confirm the beneficial effect, even for the transplantation of cadaver kidneys (8—10). In this country this has been achieved with the help of the Eurotransplant Organization, in which all transplant centers in central Europe cooperate. According to the Eurotransplant data, the difference in one year graft survival between fullhouse matches and complete mismatches is about 20% (8). This means that patients with a fully compatible graft derive true benefit from tissue typing. Because of the enormous polymorphism, however, such excellent matches are achieved only for a minority of patients, which very much limits the overall influence of HLA matching on the clinical outcome.

The fact that in most patients full compatibility cannot be achieved raises the question as to whether there is a hierarchy in the clinical relevance of antigens coded for by different loci. For nonsensitized recipients of first cadaver kidney grafts there is strong evidence that the HLA-DR antigens are particularly important (fig. 1). Therefore matching for HLA-DR is now generally accepted to rank over matching for HLA-ABC (8, 11). Of particular im-

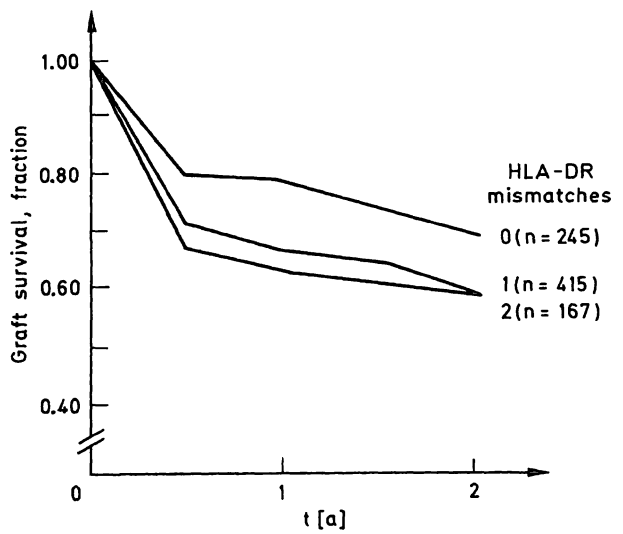

Fig. 1. Effect of matching for HLA-DR antigens on renal allograft survival in 827 patients transplanted under the auspices of Eurotransplant (8). All patients received a kidney from an unrelated cadaveric donor. Compatibility at the DR locus (group with 0 mismatches) clearly improves the graft prognosis.

portance are tissue typing and organ exchange for patients who are sensitized from a previous graft or after blood transfusions. If broadly cross-reactive antibodies are present it can be very difficult to find a cross-match negative graft at all. For all available grafts in an organ exchange network it always has first to be clarified whether or not the graft can be given to one of the highly immunized patients on the waiting list. With a suitable transplant the results in these patients are as good as in unsensitized recipients of first grafts (10).

The fact that even HLA identical living related kidney transplants can be immunologically rejected clearly points to the relevance of other antigenic systems in addition to HLA. With the exception of the ABO system these antigens are not typed for because they clearly rank below the HLA system in clinical relevance. Nevertheless a clear statistical effect has been shown for the *Lewis* antigens (12, 13) and for accumulated differences in other blood group systems (10). On the basis of animal experiments in the mouse it has to be assumed that the number of non-HLA loci which can function as histocompatibility loci is very high and probably exceeds one hundred.

For the clinical transplantation of heart and liver even matching for HLA is extremely difficult because these organs cannot be preserved sufficiently long to allow enough time for HLA typing of the donor before transplantation. In contrast to kidneys which can be preserved for up to 50 hours the conservation time for heart and liver grafts has to be below 4 hours. Because there is no artificial substitution for these organs such as haemodialysis for patients with chronic renal failure, these transplantations always have to be performed urgently. Therefore it is not possible to wait for a well matched donor.

Monitoring of Antigen-Specific T Cell Responses

Since practically all organ grafts are transplanted across some major histocompatibility barrier, graft success depends on a delicate balance between impending rejection on one side and drug-mediated immunosuppression on the other. Disturbances of this balance will either lead to graft damage by rejection processes or overimmunosuppression with the risk of severe or even fatal infection. In this situation there is an urgent need for methods monitoring the reactivity of the recipient's immune system towards the antigens of the graft.

In vitro techniques determining the reactivity of isolated T lymphocytes of allograft recipients against cultured cells of the graft donor are a suitable tool for this purpose. By this means it is possible to test whether the patient's T cell population contains antigen-reactive clones against the donor antigens and whether these clones have been stimulated or not. Relevant techniques are the mixed lymphocyte culture (MLC) and the direct and indirect cell-mediated cytotoxicity assay (direct and indirect CMC). In the MLC test the proliferative reactivity of recipient T cells indicates the presence of T cell clones predominantly directed against donor HLA-DR antigens. Information on the state of sensitisation of these clones can be obtained from the kinetics of the proliferative response. The indirect cytotoxicity assay demonstrates the presence of cytotoxic precursor cell clones to donor HLA-A, B, C antigens. Upon stimulation these precursor cells differentiate in vitro into effector cells and can be measured in a cell-mediated cytotoxicity assay (^{51}Cr release assay). In vivo sensitized cells can be measured without previous in vitro stimulation in a direct cell-mediated cytotoxicity assay using donor target cells.

For the detection of acute rejection the direct cytotoxicity assay can be expected to be the appropriate test. Indeed this assay has been demonstrated to indicate the occurrence of differentiated effector cells in blood in close correlation with the development of acute rejection episodes (15, 16). However it is disputed whether the occurrence of these cells is early enough to predict impending rejection before clinical symptoms are present. Severe limitations are the requirement for donor target cells which are difficult to maintain in the cadaver donor situation, and the complicated nature of the assay which renders it not very useful for routine purposes. Most attempts to detect activated cells by spontaneous blastogenesis of peripheral blood lymphocytes have been unsuccessful (17). A promising new approach however is the use of monoclonal antibodies, which detect differentiation antigens present only on activated T cells (see below).

During periods of clinical quiescence precursor cell assays are of particular interest because they can provide information about the risk of the patient developing T cell-mediated immune reactions. Using the indirect CMC assay it has been possible to show that a number of patients possessing cytotoxic precursor cell clones against donor antigens before transplantation loose these clones several months after transplantation (18, 19, tab. 2). Interestingly this does not happen with proliferating helper cell clones. It is not clear whether this represents clonal deletion

Tab. 2. Loss of donor-specific cytotoxic precursor cells in patients with well tolerated kidney or liver allografts (developed by about 50% of patients with a good long term result).

	T cell response to donor cells		T cell response to third party cells	
	Proliferation	Cytotoxicity	Proliferation	Cytotoxicity
Before transplantation	+++	+++	+++	+++
After transplantation	+++	Ø	+++	+++

of the cytotoxic cells, representing some type of partial tolerance, or selective inhibition of the cytotoxic clones by suppressor cells. In any case this antigen-specific defect shows that the recipient's immune system can adapt to the continued presence of foreign histocompatibility antigens. Whereas assays for rejection indicate when an increase in immunosuppressive treatment is required, studies of precursor cell activity may indicate whether treatment can be reduced or even withdrawn.

Monitoring of T Cell Subsets by Monoclonal Antibodies

The analysis of T cell subsets by monoclonal antibodies approaches the problem of monitoring T cell reactivity at a different level. This most recent diagnostic concept assumes that the development of a rejection episode and the effects of immunosuppression are reflected in the T subset composition of the blood. The subset model of the immune system originally has been based on functional tests which showed that different T cell functions, such as helper or suppressor activity or the capability to kill other cells, are mediated by distinct populations of T cells. It is now clear that these subsets represent distinct differentiation lines of T cells and differ not only in function but also in a number of cell surface proteins which can be used as subset specific markers. Monoclonal antibodies against such "differentiation antigens" have become the most important tools for the definition of subsets, their quantitative analysis and physical separation (21, 22). Furthermore it is likely that at least some T cell specific differentiation antigens are involved in the mediation of subset specific functions (23).

The chemical and functional features of six human T cell differentiation antigens defined by mouse monoclonal antibodies are summarized in table 3. Included are antigens present only on intrathymic precursors of peripheral T cells (T6), antigens presented on all peripheral T cells but not B cells (T3, T11, T12) and finally true subset specific antigens (T4 and T8). Originally T4 and T8 were thought to characterize T cells strictly on the basis of function. Recently it has become clear that they define the class of MHC antigens which restricts antigen recognition by the particular subset. T4 positive cells are restricted by class II antigens, T8 positive cells by class I antigens (24). Since the majority of class II restricted cells belongs to the helper cell pool and most class I antigen restricted cells are cytotoxic, the original association with function holds true in most instances. A schematic representation of the complex association between the specificity of MHC restriction and the function of the T4 and T8 subsets is given in figure 2. Against these and several other T cell antigens a variety of monoclonal antibodies have been produced in many different laboratories. The availability of multiple antibodies detecting the same molecule but reacting with different epitopes and possessing different functions is very useful for analytical

Tab. 3. Human T lymphocyte surface antigens defined by monoclonal antibodies (adapted from *Reinherz* et al. (23).

Antigen	M_r	Monoclonal antibodies	Expression on T cell lineage	T subset specifity
T3	20000	OKT3	all mature T cells	none
T4	55000	OKT4, Leu3, Anti-T4	majority of thymocytes and 50–60% of peripheral T cells	predominantly T helper subset (T cells restricted by class II antigens)
T6	49000	OKT6, Leu6, Anti-T6	70–80% of thymocytes but not peripheral T cells	intrathymic precursor cells
T8	76000	OKT8, OKT5, Leu2, Anti-T8	majority of thymocytes and 25–30% of peripheral T cells	predominantly cytotoxic/suppressor subset (T cells restricted by class I antigens)
T11	55000	OKT11, Leu5, Anti-T11	all thymocytes and T cells	none
T12	120000	Anti-T12	some thymocytes and all peripheral T cells	none

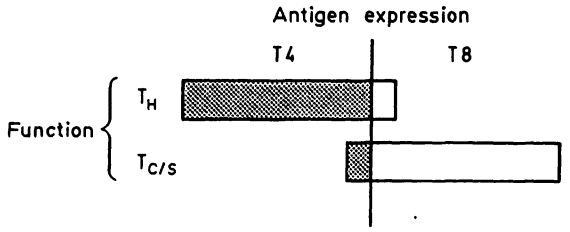

Fig. 2. Functional characterization of T cell subsets defined by monoclonal antibodies against the T cell differentiation antigens T4 and T8. The expression of T4 and T8 correlates better with the restriction of antigen recognition by MHC (HLA) class I and class II determinants than with the helper (T_H) or cytotoxic/suppressor ($T_{C/S}$) function. Most likely this good correlation is caused by a direct involvement of the T4 and T8 molecules in the mediation of restriction. Nevertheless for practical purposes they can be regarded as suitable markers of the helper and the cytotoxic suppressor subset.

MHC restriction: ☐ Class I, ▨ Class II.

purposes. An extensive comparison of T cell specific monoclonal antibodies has been preformed in a recent international workshop (25).

Monitoring relevant immunological events after organ transplantation requires serial examinations of T cell subsets in short intervals. In principle this can be performed by fluorescence microscopy. More efficient however is cytofluorography (26, 27). This permits not only the differentiation between positive and negative cells but also the study of antigen density and the correlation of fluorescent antibody staining with other cell parameters, e.g. forward and right angle scatter giving information on cell size and internal structure (26, 27). Furthermore the question, whether two or more antigens are coexpressed on the same cell can be analysed by staining with multiple antibodies conjugated with different fluorochromes (28). Relevant alterations in the T subset composition of the blood, which can occur in transplant patients, are changes in the concentration of individual subsets or the occurrence of subsets usually not present in the circulation (tab. 4).

Profound quantitative changes in the concentration of the T4 subset have been reported after immunosuppression with azathioprin, corticosteroids and antilymphocyte serum (29, 30, 31). Usually the T4 subset is markedly reduced. This indicates that this frequently used drug combination has some selectivity for helper T cells. In patients treated with the new immunosuppressive drug cyclosporine a similar drop of the T4 population has not been observed, illustrating the different mode of action of this drug. Characteristic subset patterns for the immunosuppression induced by cyclosporine remain to be established.

Tab. 4. Patterns of T subset shifts in transplant patients.

Clinical state	T cell subsets		Ratio
	T4	T8	T4/T8
Immunological quiescence	normal or ↓	normal or ↑	↓
Rejection	↑	↓	↑
Viral infection	↓	↑	↓

The diagnosis of acute rejection by subset monitoring was first discribed by *Cosimi* et al. in patients treated with conventional immunosuppression (29). They observed a sudden increase in the number of T4 cells several days before the onset of clinical symptoms. Since the ratio of the number of T4 to T8 cells is normally about 1.3 to 1.8 the change in the subset pattern elicited by conventional immunosuppression can be decribed as a decrease in this ratio below 1.0, whereas rejection is indicated by an increase to normal or above normal values (tab. 3). Since the immunosuppressive regimens used are widely different between transplant groups, it is not surprising that these findings have not been confirmed by all investigators (32). Furthermore, it has to be pointed out that there is a wide variation in the number of T4 and T8 cells between individuals. Therefore only serial examinations in the same patient during immunological quiescence and rejection are truly informative. Because the rejection-prone period is limited to the first three months after transplantation this is the most important period for these tests. The observation that the specific activation of a very limited number of clones during a rejection response can be accompanied by shifts of whole subsets is unexpected. The underlying mechanism is not understood. It's elucidation will require a better insight into the regulatory processes that maintain the normal homeostasis of the major T cell subsets in circulation.

A different type of approach to the diagnosis of rejection using monoclonal antibodies and cytofluorography is the demonstration of activated T cells in the blood. The basis for this approach is the observation that activated T cells express a variety of cell surface determinants not present on resting T cells. These activation markers include the receptor for the T cell growth factor interleukin 2, the transferrin receptor and a variety of other antigens which are not necessarily T cell specific. Many of these markers, however, are strongly expressed only during the blast stage of T cells in the lymphatic tissue, but to a much lesser degree when the activated cells enter the circulation. Nevertheless preliminary data

indicate that the measurement of activation markers can become a very powerful diagnostic tool (32, 33). The detection of subsets or activation stages of T cells normally not present in circulation is also possible by studying the coexpression of markers with two or three colour immunofluorescence (28). Thus HLA-DR antigens are usually restricted to B cells and monocytes. They can be expressed however on activated T cells. Studying the coexpression of T subset markers and HLA-DR in transplant patients, *van Es* et al. (34) have recently demonstrated an excellent correlation between acute rejection and the occurrence of cells coexpressing T8 and HLA-DR. The demonstration of activated cells in blood probably is much more sensitive than the diagnosis of rejection on the basis of subset shifts alone (32).

Viral and bacterial infections resulting from over-immunosuppression are a group of complications which are even more endangering for the transplant recipient than rejection. The diagnosis of infections is particularly difficult because they are frequently caused by agents such as the cytomegalovirus, which is not pathogenic in the immunologically uncompromised host. Furthermore the antiinflammatory effect of conventional immunosuppression obscures the typical clinical and laboratory signs. In this situation it is of particular interest that several virus infections are accompanied by a drastic increase in the T8 and a reduction in the T4 cells resulting in a reduced T4/T8 ratio (30, 34; tab. 2). Figure 3 depicts the characteristic T subset pattern of severe viral infection in one of our liver transplant patients. The infection was diagnosed on the basis of the marked increase in the T8 subset and was later confirmed by virological tests. This diagnostic aspect of T subset monitoring is probably as important as the diagnosis of rejection.

A particularly attractive trait from the point of immunosuppression is the fact that subset monitoring defines changes in T cell reactivity at a level of the immune system at which therapeutic intervention is possible. Antigen-specific types of immunosuppression so far have not been clinically successful and bear the risk of immunization instead of immunosuppression. Monoclonal antibodies against T cells and T cell subsets, however, are also potentially powerful tools for immuno-suppression. Thus antibodies against the T3 antigen and against T12 have already been used clinically and have been reported to reverse acute rejection episodes at least as effectively as corticosteroids (29, 35). Since the immunosuppression mediated by these antibodies is nonspecific, *Takahashi* et al. have used a monoclonal antibody reacting only with blast cells (36). This antibody was also effective without a demonstrable effect on normal resting T cells. This very nicely illustrates that the combined diagnostic and therapeutic use of appropriate monoclonal antibodies can open the way to an effective method of subset engineering on the clinical level.

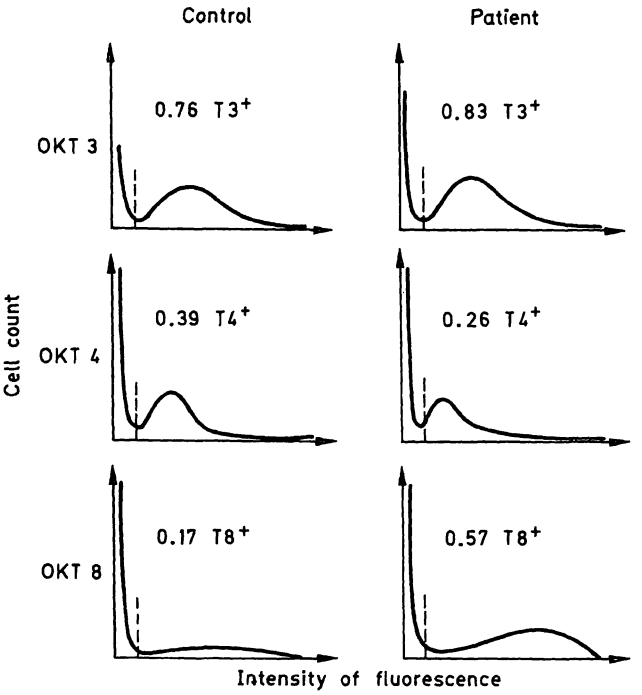

Fig. 3. Analysis of T cell subsets in the blood of a healthy control and a liver transplant patient 6 weeks after orthotopic liver transplantation, suffering from acute cytomegalovirus infection. The fraction of T cells was measured with the OKT3 antibody, the helper subset with OKT 4 and the cytotoxic/suppressor subset with OKT 8. Staining was performed in whole blood using directly labelled antibodies and the ORTHO SPECTRUM III cytofluorograph. The number of cells is plotted versus fluorescence measured in arbitrarily defined units. Cells with a fluorescence intensity above 25 units (indicated by the broken line) are regarded as positive. In the patient T4 cells are reduced and T8 cells markedly increased. This is a typical pattern for viral infections.

Conclusions

Immunological rejection elicited by the foreign histocompatibility antigens of the graft is the most important problem in clinical organ transplantation. Histocompatibility testing attempts to prevent rejection by reducing the antigenic barrier between donor and recipient. In kidney transplantation a limited but significant effect of the presently available matching techniques on the clinical outcome has unequivocally been shown. Furthermore HLA typing and extensive organ exchange represent the only possible means of finding crossmatch-negative grafts for presensitized patients with broadly cross-reacting antibodies

against HLA-A, B, C antigens. The aim of post transplant monitoring of the immune response is the diagnosis of rejection by immunological means and the recognition of states of overimmuno-suppression and infection. This will render the necessary immuno-suppressive therapy both more efficient and less dangerous. Relevant procedures are follow up studies of donor-specific immune responses and T subset monitoring in peripheral blood using T cell-specific monoclonal antibodies. The latter technique is presently in a state of rapid development and can be expected to become increasingly important. Since the presently available immunosuppressive procedures to some degree have differential effects on T subsets, this diagnostic approach deals with the immune system on a level which lends itself to therapeutic intervention. T subset-specific monoclonal antibodies are likely to become therapeutically relevant and to permit true subset engineering for the manipulation of the immune response.

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Diagnostic Sensitivity, Diagnostic Specificity and Predictive Value of the Determination of Tumour Markers[1])

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Summary: The meaningful use of tumour markers for cancer diagnosis depends on the nature of the diagnostic problem, on an adequate definition of positive and negative test results, and on the correct choice of the patient group. Potential diagnostic applications lie in screening studies, diagnosis in patients with symptoms, staging and prognosis, diagnosis of local recurrence and distant metastases, and the monitoring of radio- and chemotherapy. In quantitative tests, a positive or negative test result may be obtained by the establishment of reference ranges in control groups, or by longitudinal studies in single patients. Usually, the monitoring of therapy and the diagnosis of tumour progression are performed by following the concentration pattern of the marker. When tumour marker determinations are performed in conjunction with other diagnostic tests, the diagnostic sensitivity or specificity may be increased, depending on the particular test combination. The determination of tumour markers with limited diagnostic specificity should be performed in groups of patients with a high prevalence of the disease, e.g. in the postoperative follow-up of patients with a high risk of tumour recurrence. Tumour markers with high diagnostic specificity are also useful in differential diagnosis.

Diagnostische Empfindlichkeit, diagnostische Spezifität und prädiktiver Wert der Bestimmung von Tumormarkern

Zusammenfassung: Der sinnvolle Einsatz des Tumormarker-Nachweises im Rahmen der Tumor-Diagnostik hängt von der Fragestellung, einer adäquaten Definition des positiven bzw. negativen Testergebnisses sowie von der richtigen Wahl der untersuchten Patientengruppe ab. Unter den potentiellen Fragestellungen lassen sich Screening-Untersuchungen, die Diagnose nach Auftreten von Symptomen, Stadieneinteilung und Prognostik, Erkennung von lokalen Tumorrezidiven und Fernmetastasen sowie die Überwachung einer Radio- oder Chemotherapie voneinander abgrenzen. Der Definition eines positiven bzw. negativen Testergebnisses kann bei quantitativen Tests eine Transversal- und eine Longitudinal-Beurteilung zu Grunde liegen. Die Abschätzung des Therapieerfolgs und die Diagnose der Progredienz einer malignen Erkrankung beruht in der Regel auf einer Beurteilung des Konzentrationsverlaufs beim Einzel-Patienten. Durch eine sinnvolle Kombination des Marker-Nachweises mit anderen diagnostischen Tests lassen sich, je nach Art der Test-Verknüpfung, diagnostische Empfindlichkeit oder diagnostische Spezifität erhöhen. Die Bestimmung von Tumormarkern mit begrenzter diagnostischer Spezifität ist nur in Personengruppen mit hoher Krankheitsprävalenz, so z.B. im postoperativen Verlauf von Patienten mit hohem Rezidiv- bzw. Metastasen-Risiko, angezeigt. Tumormarker mit hoher diagnostischer Spezifität besitzen hingegen auch in der Differentialdiagnose einen Stellenwert.

Introduction

A large number of publications have appeared in recent years on the use of tumour markers in the diagnosis of malignant tumours, and for monitoring the course of malignancies. In spite of this work, there is still a lack of agreement concerning the diagnostic value of these determinations. This is connected with the fact that only relatively few investigations have taken account of the necessary criteria for the eva-

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luation of a diagnostic test. For any test, data are required on diagnostic sensitivity and specificity, on the positive and negative predictive values, together with the exact conditions under which the data are obtained. In the absence of such information, it is not possible to define those clinical situations in which the determination of tumour markers is meaningful, or to recognize those cases in which tumour markers yield no more information than already provided by established procedures. In the present account, the diagnostic value of various tumour markers is critically evaluated, taking account of the above test criteria. In this connection, particular attention is paid to published investigations, in which the diagnostic test criteria were considered, and the exact conditions of the study were reported.

Diagnostic Test Criteria, with Special Consideration of Tumour Markers

The value of a diagnostic test depends on its ability to differentiate between the clinically normal and abnormal, and between different groups of illnesses or stages of an illness. The diagnostic relevance of a test is based on the criteria of sensitivity, specificity and efficiency (1) (fig. 1). For a qualitative test, diagnostic sensitivity represents the probability that the presence of an illness will result in a positive test result, while diagnostic specificity represents the probability that the result will be negative in the absence of a particular clinical condition. The probability of achieving a correct test result in a mixed group of normal and ill subjects is called the diagnostic efficiency, and it is given by the proportion of the total test results that are correctly positive or negative. These diagnostic test parameters depend strongly on the known medical history of the investigated group of subjects. Diagnostic sensitivity is influenced mainly by the stage of the malignancy. If the stage definitions of a malignancy are well established, then the diagnostic sensitivity can serve as a useful test criterion. Diagnostic specificity, however, can vary between wide limits, depending on the type and composition of the control group.

The predictive value of positive or negative results is of particular importance in screening and in follow-up investigations. Predictive values are derived from the probability that a positive test result will truly be associated with a case of illness, or the absence of illness will be accompanied by a negative result (1). Since the prevalence of the illness is included in the calculation of the predictive value (fig. 1), the latter depends on the ratio of ill to non-ill in the investigated group. Predictive values calculated from the "Vierfelder table" are only generally applicable if the investigated population is a representative sample of the whole. For example, this may be a sufficiently large sample of patients, in which a tumour marker is determined postoperatively for the detection of tumour recurrence. In such investigations care must be taken to ensure that the respective stages of the malignancy are correctly recorded.

In quantitative methods for the determination of tumour markers, the definition of a positive test result may be based on a transverse or a longitudinal evaluation. For the transverse evaluation, it is neces-

	Disease present D	Disease absent $\bar{D}$
Test positive T	true positive = TD	false positive = $T\bar{D}$
Test negative $\bar{T}$	false negativ = $\bar{T}D$	true negative = $\bar{T}\bar{D}$

$$\frac{TD}{TD + T\bar{D}} = P(D/T) \quad \text{Predictive value pos. Test}$$

$$\frac{\bar{T}\bar{D}}{\bar{T}D + \bar{T}\bar{D}} = P(\bar{D}/\bar{T}) \quad \text{Predictive value neg. Test}$$

$$P(D/T) = \frac{P(D) \cdot P(T/D)}{P(D) \cdot P(T/D) + P(\bar{D}) \cdot P(T/\bar{D})}$$

$$P(\bar{D}/\bar{T}) = \frac{P(\bar{D}) \cdot P(\bar{T}/\bar{D})}{P(\bar{D}) \cdot P(\bar{T}/\bar{D}) + P(D) \cdot P(\bar{T}/D)}$$

$\frac{TD}{TD + \bar{T}D} = P(T/D)$ Sensitivity	$\frac{T\bar{D}}{T\bar{D} + \bar{T}\bar{D}} = P(T/\bar{D})$
$\frac{\bar{T}D}{TD + \bar{T}D} = P(\bar{T}/D)$	$\frac{\bar{T}\bar{D}}{T\bar{D} + \bar{T}\bar{D}} = P(\bar{T}/\bar{D})$ Specificity

Fig. 1. Results matrix and definitions of a qualitative test. Positive and negative predictive values are calculated with the aid of *Bayes'* theorem (48).
D: illness present $\bar{D}$: illness absent T: test result positive $\bar{T}$: test result negative (according to *Büttner* (1)).

sary to establish a reference range within a control group. The resulting diagnostic parameters depend on the composition of the control group and on the position of the cut-off point. If the cut-off point is raised, the positive predictive value increases, and there is a decrease in the proportion of false positive test results; at the same time there is a decrease in the negative predictive value, and an increased proportion of false negatives. In choosing the reference range, it must be decided whether a false positive or a false negative test result presents the greater risk for the patient. The risk depends primarily on the extent to which the therapeutic decision is influenced by the result of the individual test. For example, a "second look" operation may be performed if the concentration of a tumour marker increases postoperatively.

A quantitative test also becomes a binary test when it is used for the longitudinal evaluation of a tumour marker in individual patients. In this case, a significant increase in concentration is recorded as a positive test result. In contrast to benign illnesses, which can lead to false positive test results, the malign neoplasia shows progressive behaviour. This progression is manifested as a sustained increase in the serum concentration of a tumour marker. A longitudinal evaluation therefore permits an earlier recognition of tumour development than is possible by a transverse study. As a rule, a change in the concentration of a tumour marker in one patient has greater diagnostic sensitivity and specificity than positive or negative results obtained with the aid of a cut-off point. The problem lies in the definition of a "significant concentration increase".

In the literature there are frequent reports of the measurement of various tumour parameters in certain groups, and the result is considered to be positive if one of these test results is positive. If the correlation coefficient between the individual tests is less than unity, then such a linkage of test results indeed leads to increased diagnostic sensitivity, whereas the diagnostic specificity is decreased. Conversely, the sensitivity is decreased and the specificity increased if the declaration of a positive result requires that each of the individual tests be positive. Finally, the combined test results may be considered positive or negative when each of the tests yields a positive or negative result. The remaining cases are classified as inconclusive. In these cases, the final diagnosis must be confirmed by invasive procedures, or by further continuous observations (1, 2). The choice of test combination depends on the clinical problem. If an important therapeutic decision rests on the test result, then maximal specificity is required. On the the other hand, if all possibly affected individuals are to be detected, followed by invasive diagnosis, then the test combination should possess maximal sensitivity. Examples of both cases are described below.

Screening

Figure 2 shows how diagnostic sensitivity and specificity are influenced by the choice of patient and control groups. This example shows the changes in the sensitivity and specificity of the carcinoembryonic antigen (CEA) determination in the course of various investigations between 1969 and 1972. In this particular case, the upper limit of the reference range corresponds to the normal range of 2.5 µg/l. According to the data of *Thompson* et al. (3), the sensitivity and specificity of the CEA determination are almost perfect. During subsequent investigations, the sensitivity of the test for colorectal carcinoma decreased continually, but there was an increase in the proportion of elevated CEA values associated with non-malignant complaints of the gastrointestinal tract. It is very apparent that the reason for this phenomenon lies in the selection of the patient groups. Advanced colorectal carcinomas almost always lead to elevated CEA values. As a rule, howev-

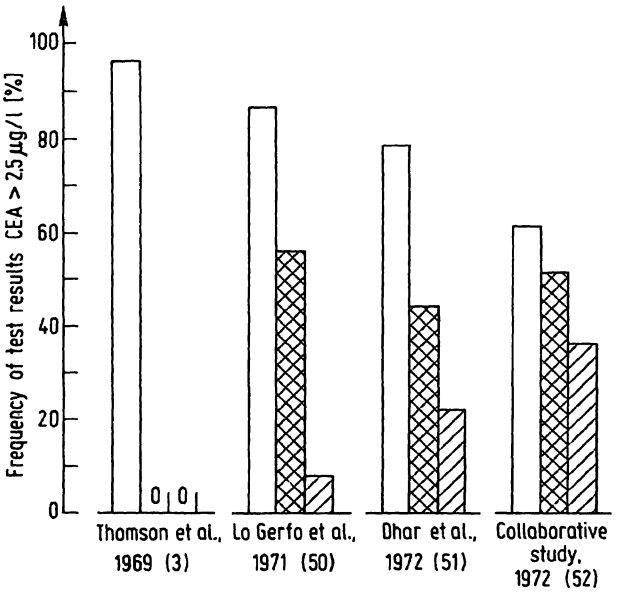

Fig. 2. Results of the CEA test from various investigations of patients with colorectal carcinomas, non-enteric cancers and non-malignant diseases of the gastrointestinal tract (according to *Bodansky* (49)).

□ colorectal cancer

▨ non-enteric cancer

▨ non-malign gastrointestinal disease.

er, non-malignant diseases of the gastrointestinal tract, e.g. liver cirrhosis, are accompanied by appreciable increases of CEA only during the active phases of the illness.

Consideration of the data on the diagnostic sensitivity and specificity of the CEA determination, together with the relatively low prevalence of colorectal carcinomas in the normal population, leads to the conclusion that the CEA determination is not suitable for screening purposes. This was confirmed in an Australian field study, which formed part of the "*Busselton* study" (4). In 1969, serum samples were taken from about 3500 inhabitants. Ninety percent of these persons continued to take part in clinical investigations at regular intervals. In 1973/74, the CEA concentrations were determined in the sera of 2372 inhabitants, aged 40 and older. The sensitivity of the CEA determination, i.e. the frequency of positive results for patients with malignant tumours, was 27%, while the specificity was 97%. Since the prevalence was 1.4%, a positive predictive value of 0.4% can be calculated, i.e. only one in 250 persons with a positive test result actually had a malignant tumour. Since the CEA determination detects advanced rather than early tumour stages, it is doubtful whether a positive CEA result could have been followed by effective curative therapy. In summary, the CEA determination is therefore not suitable for screening for malignancies in a normal population. Similar considerations apply to other tumour markers, whose diagnostic sensitivity and specificity are comparable to those of CEA. The CEA determination is also unsuitable in a risk group, e.g. hospital patients (5) or subjects with polyps of the large intestine (6) or with colitis ulcerosa (7). This is because an increased prevalence of disease with a possible increase in diagnostic sensitivity is accompanied by a decrease of diagnostic specificity.

The only tumour marker that can possibly be considered as suitable for screening purposes is alpha-foetoprotein (AFP). Taking into account the time course of the concentration, and by choosing a suitable cut-off point, AFP measurement is relatively specific for liver cell carcinoma (8). In addition, patients with liver cirrhosis represent a risk group in which the prevalence of hepatocellular carcinoma is about 5% according to clinical studies, or 10–25% according to autopsy studies (9). Diagnosis of liver cell carcinoma by serum AFP measurement is, however, made difficult by the occurrence of intermittent, short term concentration increases, which are unrelated to the presence of carcinomas (10). These short term increases do not, however, normally exceed 2000 µg/l. In patients without hepatoma, the concentration then decreases with a half life of 5–6 days, which corresponds to the normal half life of serum AFP. In contrast, patients with liver carcinoma show a further increase. A positive test result can therefore be defined as follows: the AFP concentration must either exceed 2000 µg/l, or lie above 50 µg/l with subsequent increases in the course of the disease. Thus, *Lehmann & Wegener* (10) reported that 24 out of 27 cases of hepatocellular carcinoma in a patient collective were diagnosed by AFP determination (89%), 17 of these before the appearance of symptoms (63%).

Differential Diagnosis after the Appearance of Symptoms

For various reasons, CEA determination plays a secondary role in the differentiation of malignant tumours. Colorectal carcinoma must most frequently be differentiated from benign illnesses of the gastrointestinal tract; but these same benign illnesses are accompanied by a relatively high frequency of false positive increases in CEA concentration (11). This means that the CEA test is not sufficiently specific to serve as a basis for differential diagnosis. By increasing the upper limit of the reference range, the specificity of the diagnosis can be increased, but the detected tumours are then predominantly in an advanced stage. The CEA test is too insensitive for the detection of localized carcinoma of the stomach, mammary gland, bronchial tract, ovary and cervix (11, 12).

Despite the relatively low diagnostic sensitivity of the CEA determination, it may be of value in the differential diagnosis of those tumours that are slow to produce symptoms, and which cannot be recognized by present diagnostic procedure until a late stage of development. This is the case for carcinoma of the pancreas. The range of CEA concentrations in diagnosed pancreatic carcinoma is significantly different from that found in non-malignant diseases of the pancreas and gastrointestinal tract (13). For the CEA determination to be of special diagnostic value, however, its sensitivity and specificity should be higher than those of other established procedures. In a double blind study, the value of the CEA determination in differential diagnosis was studied in patients with suspected pancreatic carcinoma, where immediate confirmation of the diagnosis had not been possible (13) (tab. 1). In three out of nine cases with later confirmed pancreatic carcinoma, CEA concentrations were markedly elevated on admission to hospital. In these three cases, however, the diag-

Tab. 1. Initial plasma CEA level and interval before definite diagnosis, in patients suspected of carcinoma of the pancreas (according to the MRC Tumour Products Committee (13)).

Definite diagnosis							
Pancreatitis or gallstones		Carcinoma of the pancreas		Other malignancy		Other non-malignant disease	
Days to diagnosis	Initial CEA (µg/l)	Days to diagnosis	Initial CEA (µg/l)	Days to diagnosis	Initial CEA (µg/l)	Days to diagnosis	Initial CEA (µg/l)
1	13	3	100	7	17	2	23
11	21	7	50	7	82	14	14
120	17	8	191	461	26	23	33
123	8	18	32	un-	36	379	9
164	11	28	14	known			
193	6	28	10				
215	11	90	21				
		230	11				
		956	12				
n	7		9		4		4

n = Total patients

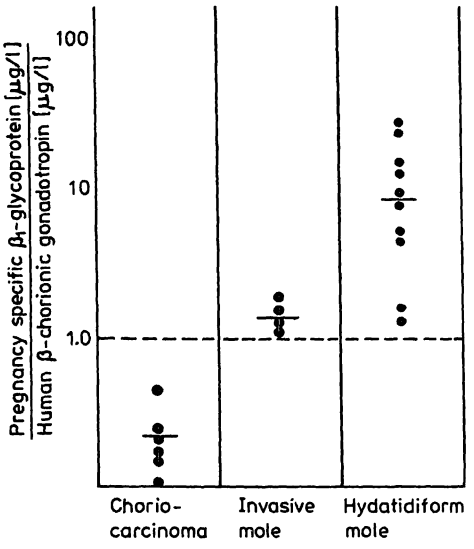

Fig. 3. Ratio of serum pregnancy specific β_1-glycoprotein to human β-chorionic gonadotropin in patients with chorioncarcinoma, invasive mole and hydatidiform mole. The quotient was calculated from the concentrations in µg/l (according to *Sakuragi* (17)).

nosis was confirmed within a week by other established procedures. In those cases that were not diagnosed until they were more advanced, the CEA determination was not sensitive enough for a significantly earlier diagnosis. In contrast, the measurement of CEA is relatively sensitive for the diagnosis of medullary thyroid carcinoma, although the determination of calcitonin is more sensitive and more specific. Since there is a good correlation between these two tests, the CEA determination does not appear to be capable of yielding any essentially new information (14).

As already mentioned, the determination of AFP is a useful aid to the diagnosis of hepatocellular carcinoma. On the other hand, germ cell tumours of the testes are diagnosed clinically by the local swelling. The specific determination of human chorionic gonadotropin (HCG) has long been used for the diagnosis of hydatidiform mole and chorioncarcinoma (15). These two malignancies can be differentiated from one another by the determination of pregnancy-specific β-1 glycoprotein (SP-1) and HCG (16) or the β-subunit of HCG (17) (fig. 3). In chorioncarcinoma, the concentration of HCG-β- is higher than that of SP-1, whereas the converse is true for hydatidiform mole. The fact that the SP-1/HCG-β ratio increases during pregnancy indicates that the change in ratio is the result of an altered differentiation of the cells of the trophoblast (17).

Staging, Diagnosis of Metastases, Prognosis

Determination of the stage of neoplastic development and the diagnosis of metastases may be based on the result of one type of test, or on a combination of various diagnostic procedures. In addition, the time course of the concentration of a tumour marker following therapeutic intervention provides a matrix of results. The use of tumour markers for staging and for the diagnosis of metastases is closely related to their importance in prognosis.

In patients with colorectal carcinoma, there is a significant positive correlation between the preoperative CEA concentration and the spread of the carcinoma. Between *Dukes'* stages A-D, there is an increase in the concentration of CEA and an increase in the proportion of cases showing elevated CEA. Thus a relationship exists between preoperative CEA concentrations and the prognosis. The higher the concentration range, the higher the proportion of patients with tumours in an advanced stage of development (20–22). According to recent studies, however, CEA concentrations within each *Dukes'* stage have little or no additional prognostic importance (23–25). By determination of the time course of CEA concentrations directly after surgical removal of colorectal carcinomas, we were able to distinguish between patients with CEA-secreting residual tumours and those in which CEA-producing tissue had

been removed, or in which no preoperative CEA increase had been found (26). It was shown that CEA concentrations greater than 20 µg/l can also be caused by the resectable primary tumour. We concluded from this that, after elimination of patients with CEA-producing residual tumours, even a high CEA serum concentration need not mean a poor prognosis. This relationship has meanwhile been confirmed by *Persijn & Hart* (24). If cases revealing a residual tumour during operation are excluded from the follow up, there is no longer a significant correlation between the preoperative CEA concentrations and the frequency of tumour recurrence. For tumours sited away from the rectum or colon, however, CEA concentrations 8–10 fold higher than the upper limit of the normal range indicate, with a high degree of certainty, the presence of an extensive, non-operable residual tumour or distant metastases (27, 28).

Measurement of CEA can serve as a valuable supplement to other procedures for the diagnosis of metastases. In this connection, one study should be noted in which the combination of CEA measurement and liver scintigraphy, showed a markedly higher positive predictive value than the single test (29) (fig. 4). The result was counted as positive if both tests were positive. The positive predictive value of scintigraphy was 75%, and that of the CEA determination was 64%, whereas the combination of the two resulted in a value of 100%. It is to be expected that the diagnostic sensitivity of such a combination will be lower than that of the single test. Thus, the diagnostic sensitivities are 42.1% + 14.0% = 56.1% for the CEA determination, and 42.1% + 36.8% = 78.9% for liver scintigraphy, but only 42.1% for the test combination (fig. 4). The consequence of combining individual tests in this way is that fewer false diagnoses are made, but the diagnosis at first remains uncertain for a larger number of patients. These uncertain diagnoses must then be resolved by further time course observations and/or alternative diagnostic procedures.

Preoperative measurement of AFP and HCG makes a decisive contribution to the clinical staging of non-seminomatous germ cell tumours of the testes (30). The importance of the determination of these tumour markers is, however, relative, if staging is based on pathological-anatomical criteria (31). This relationship is shown in table 2, where it is seen that staging was improved by the determination of markers only in stage A. As the tumour progressed further, all cases showed tumour-associated increases in the concentration of the tumour markers. On the other hand, the AFP and HCG concentrations are relatively insensitive indicators in the early stages of lymph gland metastasis.

The existence of residual tumours and/or distant metastases, following therapeutic intervention, can also be diagnosed by monitoring the time course of the concentration of a tumour marker, providing the

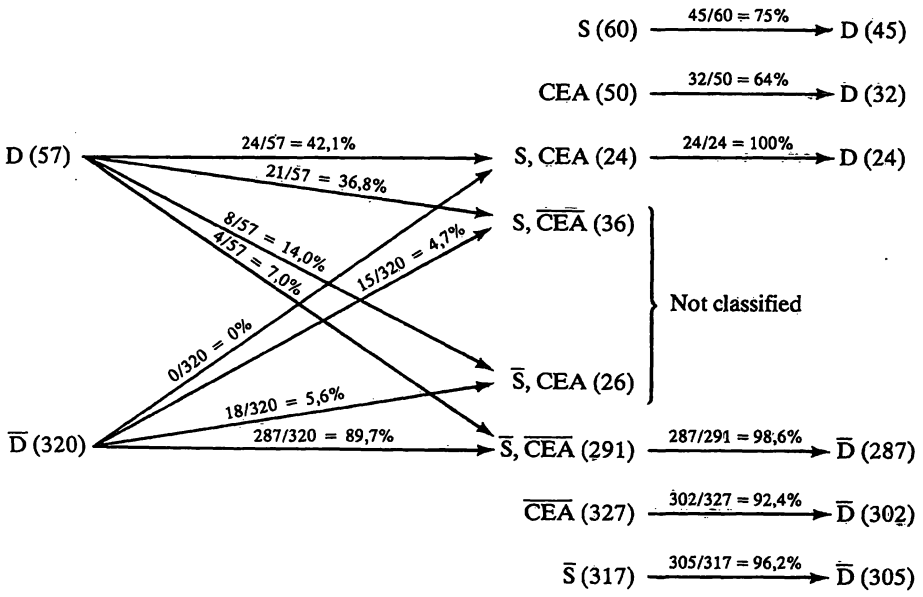

Fig. 4. Diagnosis of liver metastases by liver scintigraphy (S) and CEA measurement (CEA). The upper limit of the CEA reference range was 3.6 times higher than the upper limit of the normal range. The presentation is according to *Büttner* (2), using data from *McCartney & Hoffer* (29).
D: liver metastases confirmed in course of the illness. $\overline{D}$: no liver metastases in follow-up period. S: positive scintigram $\overline{S}$: negative scintigram CEA: positive CEA test $\overline{CEA}$: negative CEA test.

Tab. 2. Frequency of elevated levels of human chorionic gonadotropin (HCG) and alpha-foetoprotein (AFP) in serum samples obtained after orchiectomy but immediately before any other therapy in 142 patients with non-seminomatous germ cell tumours[a] (according to *Skinner & Scardino* (31)).

Stage[b]	No. of patients	HCG (%)	AFP (%)	Either (%)
A	67	7	9	10
B1	15	29	33	50
B2	23	52	43	64
B3	6	83	75	100
C	31	84	60	93

[a] These figures represent data collected since 1973 from a combined group of patients treated at the Walter Reed Army Medical Center, the National Cancer Institute and UCLA.

[b] Stage A: disease confined to the scrotum.
Stage B1: metastases to <6 retroperitoneal nodes with no node >2 cm in diameter.
Stage B2: metastases to ≥6 retroperitoneal nodes or any metastases >2 cm in diameter.
Stage B3: bulk abdominal disease.
Stage C: metastases above the diaphragm or to the viscera (liver).

same marker was elevated before the onset of therapy. For tumour markers with a known biological half life and a uniform pattern of elimination, a delayed decrease can be distinguished from a non-delayed decrease in concentration. At the moment this is possible only for AFP (32). For other markers, a complete fall in concentration is generally considered to be a negative result, and an incomplete decrease is counted as positive.

Contradictory data have been reported for the specificity of the post-therapy diagnosis of residual tumours and/or distant metastases by monitoring the time course of tumour markers. Many authors consider a complete or non-delayed decrease in marker concentration to be relatively certain evidence for the complete elimination of tumour cells (32–34). According to other studies, however, the diagnostic specificity of a non-delayed or complete concentration decrease is relatively low (35–37).

A decrease in CEA concentration to a stable but elevated level may be observed when a non-malign illness is also present (36). The absence of any decrease, or an incomplete decrease of CEA with a subsequent rise is, on the other hand, relatively certain evidence of a residual tumour (33). There is a lack of agreement on the diagnostic value of an extended AFP half life (32, 35).

Diagnosis of Tumour Recurrences

After resection of a carcinoma, the rationale of recognizing renewed tumour growth is similar to that of a screening investigation in a highly selected population with a high frequency of illness; in patients with resectioned colon carcinoma, for example, this frequency is 30 to 40%. On the basis of this high frequency of illness, the predictive value of a positive tumour marker test is markedly higher than in a normal population or in patients with questionable diagnoses. The result may be based on an individual test or a combination of different tests, depending on the clinical problem and the therapeutic consequences. In both cases, the decision as to whether a result is positive or negative depends on the reference range, which is established by a transverse study. Alternatively, a result may be designated negative or positive on the basis of the time course of the marker concentration in one particular patient. In this connection, for the purpose of comparing different investigations, it is essential to establish the exact criteria for a concentration increase.

The technique used by *Martin* et al. (38) for the determination of a CEA increase takes into account the variance of the method. Intra- and interassay variance of the CEA determination were determined in 9 different concentration ranges between 1 and 10 µg/l. These values were recorded on a nomogram with calibration marks for the average value and the 95% confidence interval. If a significantly increased value is measured, then both samples are analysed again in a single assay. Thus, alterations of concentration due to methodical variance are largely excluded. Alterations of CEA concentration caused by non-malign conditions are not, however, excluded by this method.

Steele et al. (25) estimated the CEA increase by a slope analysis, using at least four determinations to calculate the slope. The logarithms of CEA concentrations are plotted against time, and the average monthly concentration increase can be calculated from the slope of the regression line. This method permits an exact retrospective, statistical evaluation, and provides a quantitative assessment of malignant growth. For diagnostic purposes and for therapeutic decision making, however, a relatively long time is required to obtain the positive test result. Other workers regard the result as positive if at least two successive analyses show concentrations outside the normal range (39), or if one value is several fold higher than the upper limit of the normal range (40). More complicated definitions of a concentration increase are also used (21, 24).

The diagnostic value of a postoperative CEA increase can be assessed from the results of two collaborative studies using a large number of cases, shown figure 5. Despite different working definitions for the positive test result, the results of the two studies compare well with each other. The study represented in figure 5a was based on the average monthly increase in CEA (25), and was limited to patients at *Dukes'* stages B2 and C, i.e. a high risk group for tumour recurrence. A positive test result was recorded if the average CEA increase was greater than 3% per month. The diagnostic parameters are calculated from the "Vierfelder table". At 66.4%, the positive predictive value of the CEA increase is relatively low, especially if the possibility of a second-look-operation is considered. In the quoted study, the positive predictive value was, however, markedly decreased by basing a positive test result on a single CEA concentration, instead of a concentration increase. The predictive value was decreased, even if the elevated concentration was more than four fold higher than the upper limit of the normal range.

In the study represented in figure 5b, the test result was positive if at least one postoperative CEA value was 3.2 times higher than the upper limit of the normal range (40). The number of cases is similar to that in the previous investigation. Here also, the pre-

a

	Recurrence	No recurrence	
Monthly rise >3%	104	52	$P(\bar{D}/T) = \frac{104}{156} = 66{,}7\%$
Monthly rise ≤3%	55	388	$P(\bar{D}/\bar{T}) = \frac{388}{443} = 87{,}6\%$
	$P(T/D) = \frac{104}{159} = 65{,}4\%$	$P(\bar{T}/\bar{D}) = \frac{388}{440} = 88{,}2\%$	$\frac{TD + \bar{T}\bar{D}}{N} = \frac{492}{599} = 82{,}1\%$

b

	Recurrence	No recurrence	
CEA >40 µg/l	70	32	$P(D/T) = \frac{70}{102} = 68{,}6\%$
CEA ≤40 µg/l	38	328	$P(\bar{D}/\bar{T}) = \frac{328}{366} = 89{,}6\%$
	$P(T/D) = \frac{70}{108} = 64{,}8\%$	$P(\bar{T}/\bar{D}) = \frac{328}{360} = 91{,}1\%$	$\frac{TD + \bar{T}\bar{D}}{N} = \frac{398}{468} = 85{,}0\%$

Diagnostic sensitivity of the CEA-rise as first indicator of tumour progression:

$$P(T/D) = \frac{58}{108} = 54\%$$

Fig. 5. Diagnostic sensitivity, specificity and efficiency, and the predictive value of the CEA concentration rise for the recognition of tumour recurrence in patients with colorectal carcinoma.
a) Data from *Steele* et al. (25) for patients with colorectal carcinoma at *Dukes'* stages B_2 and C.
b) Data from *Tate* (40).

dictive value of the positive test result is lower than 70%, whereas the predictive value of the negative test result is approximately 90%. For more than half the patients with tumour recurrence, the elevated CEA concentration was the first indicator that the disease was still present.

There is no disagreement that the increase of CEA in a certain percentage of the patients is an earlier indicator of relapse than other diagnostic measures or subjective symptoms. With few exceptions, no causal therapy is so far available for distant metastases of colorectal carcinoma. The time between an increase of CEA concentration and the diagnosis of a local recurrence is therefore of special importance. A local recurrence is more amenable to surgery than distant metastases. According to the data of *Persijn & Hart* (24), about one quarter of local tumour recurrences were first detected by an increased CEA concentration.

The question arises as to whether a second-look-operation should be performed with the aim of resectioning the residual tumour, solely on the basis of an increased CEA concentration. In other words, what is the predictive value of the CEA concentration increase as the first objective indication of a local tumour recurrence in the absence of distant metastases? The most optimistic study reported so far is by *Minton & Martin* (41). In 40 patients showing preoperative elevated CEA concentrations, which became normalized after surgery, a second-look-operation was subsequently performed solely on the basis of an increased CEA concentration. CEA was determined at three monthly intervals in 22 patients, and at monthly intervals in 18 patients. The concentration increases were confirmed by use of the previously mentioned nomogram. The positive predictive value of a CEA increase for a local relapse was 48%. In other studies, the predictive values for a local relapse are, however, much lower. The value of 7% from the investigations of *Mach* et al. (42) is comparable with the results of *Persijn & Hart* (24). In the latter study, an increase of CEA at least 200 days before diagnosis of a local relapse showed a predictive value of 5%. The positive predictive value for a local relapse may possibly be increased by slope analysis (43). Distant metastases are more frequently accompanied by steep concentration increases, whereas the increase for a local relapse tends to be more gradual. In a comparison of patients with slow and steep CEA concentration increases, *Wood* et al. (39) reported twice as many cases of relapse in the former group.

If, as in the last example, a serious therapeutic decision must be made on the basis of a single diagnostic test, then the predictive value of the positive test result becomes a factor of major importance in the estimation of the diagnostic value. In this case, the proportion of false positive test results is critical. If, however, a test or test combination is required that will detect the greatest possible proportion of patients with tumour recurrence, then the negative predictive value is critical. In the latter situation, the proportion of false negative test results should be kept as low as possible. On the other hand, the false positive results can be tolerated, because the diagnosis can be confirmed by invasive procedures, or by a more exact monitoring of the patient. This is demonstrated below by an example (tab. 3; (44)). A test combination is required for the detection of distant metastases, following the operation for mammary carcinoma. The tests should impose as little strain as possible on the patients, and be capable of the earliest possible detection of the highest possible percentage of distant metastases. Table 3 shows the results of individual tests in cases of diagnosed distant metastases. With respect to diagnostic specificity, the CEA determination is superior to all other methods. At least one of the tests marked with an asterisk was positive for 46 out of 47 patients with distant metastases. In one half of the cases, the positive test result was obtained 3 months or more before the conclusive clinical diagnosis. The and/or linkage of individual tests should result in a relatively low specificity for the positive test result. Nevertheless, false positive results were recorded for only 3.5% of the patients. There are two reasons for this:

1. the upper limit of the CEA reference range was three times higher than the normal range, and
2. the group in question is a very high risk group with a metastasis prevalence of 33%.

Tab. 3. Abnormal biochemical and physical tests of first presentation with metastases in 47 patients with breast cancer (according to *Coombes* et al. (44)).

Test	No. abnormal
Biochemical tests	
Erythrocyte sedimentation rate	5/31 (16%)
γ-Glutamyl transpeptidase	9/30 (30%)*
Alkaline phosphatase	15/46 (32%)*
CEA	16/36 (44%)*
Hydroxyproline	8/32 (25%)
Physical tests	
Clinical examination	19/47 (40%)*
Chest X-ray	19/44 (43%)*
Bone scan	15/44 (34%)
Liver scan	3/30 (10%)
Liver ultrasound	4/26 (15%)
Skeletal survey	13/38 (34%)
Bone-marrow aspirate	7/34 (21%)

* Combination of these tests would have detected metastases in 46/47 patients.

Monitoring of Radio- or Chemotherapy

Tumour markers are frequently the most sensitive objective parameters for detecting the presence of marker-producing tumours or portions of tumour tissue that have resisted radio- or chemotherapy. In patients with cervical carcinoma, providing the CEA serum concentration is elevated before the start of radiotherapy, the success of the treatment can be monitored by measuring the time course of the CEA concentration (28). Determination of CEA provides an objective assessment of the resistance to chemotherapy of CEA-producing mammary carcinomas (about 50% are CEA producers) (45, 46). The chemotherapy of non-seminomatous germ cell tumours is monitored by the determination of AFP and HCG. In the quoted example (47) (fig. 6), there is a dramatic fall in the AFP concentration at the start of chemotherapy, with a less pronounced decrease in HCG. Tumour recurrence during chemotherapy is detected earlier by the occurrence of increased marker concentrations than by other diagnostic procedures. This example shows that a decrease in the concentrations of a tumour marker during chemotherapy only reflects the chemotherapeutic sensitivity of a corresponding proportion of marker-producing tissue. Unchanged concentrations of marker do not exclude the possibility of the further growth of marker-negative tissue. On the other hand, increasing concentrations are usually an indication of continued or renewed tumour growth.

Resumé

The effective use of tumour marker determinations in tumour diagnosis depends on an adequate definition of the positive or negative test result, and on the correct choice of the patient group to be investigated. The diagnostic potential of these tests should be suited to the nature of the clinical problem. If they are used in appropriate combinations with other diagnostic procedures, and the time course of marker concentration is taken into account, then tumour markers can extend and expand the possibilities for tumour diagnosis. Tumour markers are particularly effective in the early recognition of local relapses and metastases, and for the sensitive and objective monitoring of radio- or chemotherapy.

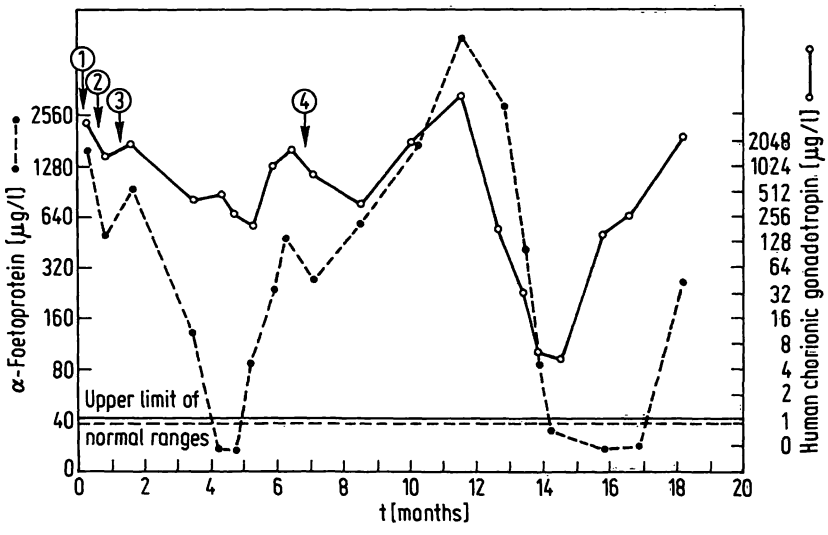

Fig. 6. Time course of serum concentrations of alpha-foetoprotein and human chorionic gonadotropin in a patient with non-seminomatous germ cell tumour (from *Lange* et al. (47)).
(1) Radical orchiectomy,
(2) retroperitoneal lymphadenectomy,
(3) start of chemotherapy,
(4) change of chemotherapeutic regime

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