Detection of AFP by radioelectrocomplexing*

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Immunological methods based on the determination of the primary interaction between antigen and antibody are more sensitive for the detection of molecules such as alpha-foetoprotein (AFP) than those in which secondary interactions are measured. Radioimmunoassay (RIA) procedures, which are among the most sensitive, have been applied to AFP quantification (see Masseyeff, Gilli and Bonet, this symposium). All RIA procedures involve the two stages of immune complex formation and separation of free from bound radiolabelled antigen. Most have limitations due to the time taken in the incubation or separation processes, or to the need for centrifugation, washing and other steps which cannot easily be automated. An ideal RIA would be one which is:

(1) sensitive
(2) rapid
(3) simple to perform (not requiring washing or centrifugation)
(4) safe to perform (minimum total radioactivity; minimum risk of splashing or spillage)
(5) simple to interpret
(6) quantitative
(7) reproducible
(8) immunologically specific (yet able to reveal non-specific effects such as radiation damage)
(9) capable of detecting both antigen and antibody without change of conditions
(10) capable of identifying cross-reacting antigen-antibody systems, and of quantitating the degree of cross-reaction
(11) capable of showing no labelled antigen in the 'bound' antigen fraction in the absence of antibody
(12) capable of showing 100% of the labelled antigen in the 'bound' antigen fraction in the presence of excess antibody

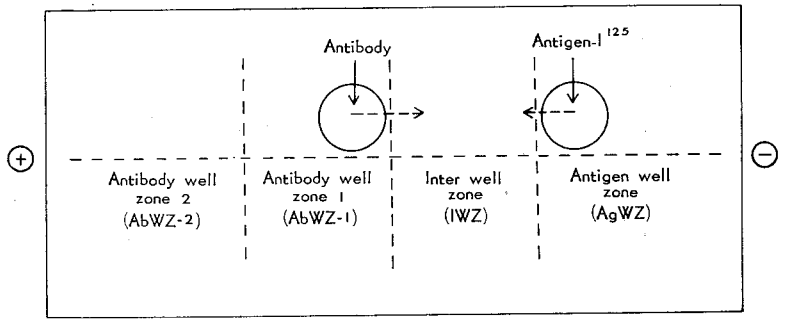
* Presented at the First Meeting of the International Research Group for Carcinoembryonic Proteins, Sapporo, Japan, 1973. This paper was written after the Symposium. Consideration has been given to some of the questions raised in discussion of the verbal presentation.

(13) uncomplicated by prozone phenomena
(14) conservative of reagents
(15) inexpensive
(16) suitable for testing of undiluted samples
(17) adaptable to microscale
(18) adaptable to testing of large numbers of sera
(19) adaptable to automation

The RIA to be described, called radioelectrocomplexing (REC–1) is a development of the technique of counter-immunoelectrophoresis (CIE-for the application of CIE to AFP detection see Kohn, this symposium). REC involves the formation of sub-visible complexes of radiolabelled antigen with antibody as a result of counter-directional movement produced during gel electrophoresis. In REC the two stages of radiolabelled antigen-antibody binding, and separation of free from bound antigen, are achieved by a single process of electrophoresis.

AFP fulfills the four requirements for detection by REC: it is available in a purified form, it can be radiolabelled, it is immunogenic, and it has an anodal migration on electrophoresis. AFP–I^{125}, AFP 'standard' solutions, and anti-AFP serum were obtained commercially (α-feto-125 kit, Dainabot Radioisotope Laboratories, Tokyo, Japan; ULTA, Zaragoza, Spain). Microscope or lantern slides were coated with 1% agar in veronal buffer (pH 8.6; r/2=0.05) to a depth of 2 mm (4 ml and 25 ml respectively). Wells (3 mm diameter) were punched in pairs (4 mm interwell distance), 4 per microscope slide, 15 per lantern slide. AFP–I^{125} and dilutions of anti-AFP (each 5 μl) were pipetted into the cathodal-end (antigen) and anodal-end (antibody) wells respectively. After electrophoresis (60 min.; 2.5 mA/reaction), the agar was cut using a 6 inch scalpel blade, vertically to divide the gels into 4 zones within each reaction pair (Table 1: antigen well zone-AgWZ; inter-well zone-IWZ; gel surrounding the antibody well and 2 mm anodal to the well-AbWZ–1; remaining gel-AbWZ–2) and horizontally between each pair of wells. In the absence of antibody to AFP, labelled AFP migrated anodally out of the AgWZ through the IWZ and AbWZ–1, and into AbWZ–2. When anti-AFP was present, AFP–I^{125} became bound to cathodally moving antibody. The distribution of radioactivity was related to the titre of anti-AFP. With high titre anti-AFP, a proportion of the radiolabel was confined to the AgWZ. With medium titre antisera the majority of the radiolabel was present in the IWZ. When anti-AFP was tested at dilutions near antibody extinction radioactivity was present not only in the IWZ but also in the AbWZ–1. Thus, unlike the situation in CIE where the zone of visible antigen-antibody reaction is confined to the

Table 1. *Distribution patterns of AFP–I^{125} when migrated against dilutions of anti-AFP serum*



Anti-AFP Dilution	AbWZ–2	AbWZ–1	IWZ	AgWZ	Total cpm
2^{0}	0	0	96	4	3275
2^{-2}	0	0	97	3	3158
2^{-4}	0	0	95	5	3350
2^{-6}	0	0	96	4	3420
2^{-7}	0	0	94	6	3119
2^{-8}	11	9	74	6	3367
2^{-9}	58	29	8	5	3460
2^{-10}	82	12	2	4	3201
2^{-11}	91	4	2	3	3199
2^{-12}	93	2	1	4	3310
C	94	1	1	4	3385

IWZ, in REC the reaction zone (RZ) or zone of binding is extended to include AgWZ and AbWZ–1. Anti-AFP antibody titre could be quantified by expressing the proportion of radioactivity in the RZ as a percentage of the total (see Table 1). Conditions of gel composition and concentration, electrical current, duration of electrophoresis etc. were chosen such that immune complex formation was maximized and dissociation minimized[1].

AFP was detected by inhibition of the direct REC reaction. Anti-AFP at a dilution which bound 30–40% of the AFP–I^{125} was pipetted into the antibody well and amounts of standard AFP (5 μl) were delivered into the cathodal-end (antigen) well. Counter-directional movement was initiated by a short period of electrophoresis (10–15 min). After addition of AFP–I^{125} to the antigen well, electrophoresis was continued for 60 min. The rationale of the initial electrophoresis was to allow binding to occur between unlabelled AFP and anti-AFP, thereby maximizing the probability of com-

petitive inhibition of binding between the subsequently added AFP-I^{125} and reagent anti-AFP.

Figure 1 shows a typical curve of binding inhibition produced by an AFP standard solution, other aliquots of which have been tested by Dr. H. Hirai. In this experiment the RZ was taken as the AgWZ+IWZ. In some experiments AFP at concentrations of 2.5 ng/ml significantly inhibited binding. In routine testing, confidence can be placed in levels greater than 10 ng/ml.

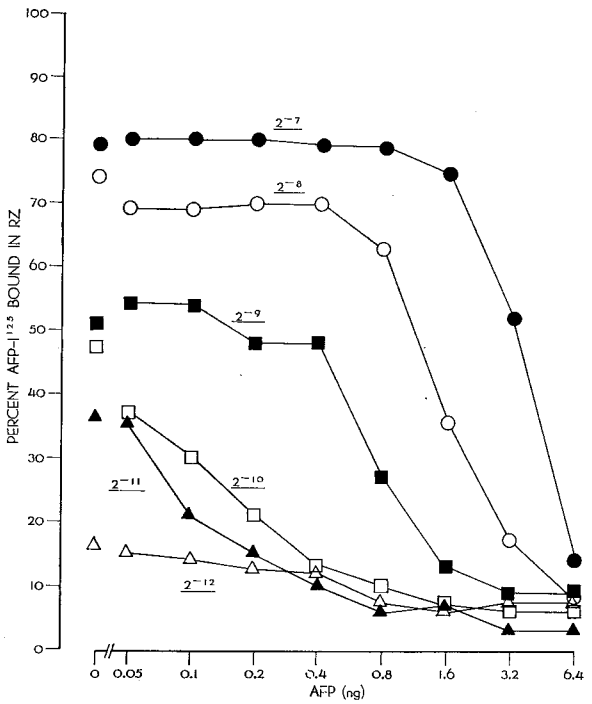


Fig. 1. REC assay of AFP-I^{125} with anti-AFP (ULTA): inhibition by AFP standard (Dainabot). (# 498,500)

The REC assay has been used to test the sera of the last 139 Singapore patients with hepatocellular carcinoma (HCC) who were diagnosed on the basis of a positive reaction for AFP on immunodiffusion (93 patients), or on histopathological grounds (46 patients - AFP negative on immunodiffusion). 52 patients, in whom the diagnosis of HCC was suspected but no evidence of malignancy was seen on histological examination of biopsy tissue, and who were AFP negative on immunodiffusion, were also tested. The results are shown in Table 2. It can be seen that 11 (7.9%) of 139 HCC patients had AFP levels of 10 ng/ml or less. This proportion compares

Table 2. *AFP levels in singapore patients with HCC and in a comparison group*

AFP (REC) (ng/ml)	HCC			Non-HCC AFP (ID)−ive (52)
	AFP (ID)+ive (93)	AFP (ID)−ive (46)	Total (139)	
<5	0	6	6 (4.3%)	20 (38%)
5–10	0	5	5 (3.6%)	13 (25.5%)
20–160	0	10	10 (7.2%)	6 (11%)
320<	93	25	118 (84.9%)	13 (25.5%)

well with the results obtained by Nishi and Hirai[2] of 11% (7/56) with levels less than 20 ng/ml and by Ishii[3] of 7% (8/107) with AFP levels less than 15.6 ng/ml. Using the same conservative criteria of positivity (>10 ng/ml) 35 (76%) of 46 of the patients negative for AFP on immunodiffusion had elevated levels, as did 19 (36.5%) of 52 of the HCC-suspected but not histopathologically proven patients.

At the University of Malaya, Kuala Lumpur, Dr. P. Chan (Dept. of Bacteriology) and Dr. T. K. Ti (Dept. of Surgery) have adopted REC for their studies on AFP levels in patients with liver diseases. They have found REC to be a simple procedure for quantitation of AFP levels greater than 10 ng/ml. Interestingly, among nonhepatocellular carcinoma patients they are finding fewer with levels $\geq$10 ng/ml than in the Singapore study[4].

In 1972 Waldmann and McIntire[5] reported that patients with ataxia-telangiectasia appeared to be unique among those with congenital immune deficiency diseases in that they had raised levels of AFP. Ataxia-telangiectasia is a rare disorder characterized by cerebellar ataxia, oculocutaneous telangiectasia, recurrent sino-pulmonary infections and a tendency to the development of lymphoid malignancy. An immune deficiency, invariably involving thymic dependent cell-mediated immunity and frequently also affecting the humoral immune system, underlies the recurrent infections. A range of other disorders, including gonadal and hepatic dysfunction, have also been described.

Eight patients with ataxia-telangiectasia from three families have been under study at the Royal Children's Hospital in Melbourne, Australia. Sera from seven of the eight were sent as coded samples to Singapore, together with sera from two parents, two normal siblings of one of the families, and twelve other sera, mainly from children with immune deficiency diseases. All seven of the ataxia children had elevated AFP levels between 40–320 ng/ml[6]. Both parents and both of the normal siblings had normal

levels (<10 ng/ml), a finding in keeping with that of Waldmann and McIntire. These authors suggest "that the findings support the hypothesis that the primary abnormality of patients with ataxia-telangiectasia is a defect in tissue differentiation. This abnormality may be due to the defective interaction between the entodermal and mesodermal germ lines, an interaction that seems to be required for the differentiation of gut-associated organs such as thymus and liver".

Finally, in view of the interest in AFP levels and hepatitis B antigen (HB Ag) in HCC patients, sera from 40 blood donors positive for HB Ag by CIE in 1% agarose were tested for AFP. None of the 40 had levels greater than 10 ng/ml. However, variation in the distribution patterns of AFP–I^{125} suggested that differences in levels between 1.25 and 10 ng/ml may exist. This possibility is presently being investigated.

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