MECHANISMS IN THE INDUCTION OF UNRESPONSIVENESS TO SKIN ALLOGRAFTS IN THE MOUSE

Simon P. M. Lems

Front cover illustration by **Margriet Lems**

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
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Aan Barbara en onze kinderen
Margriet, Floris, en Nicolien.

Aan onze ouders.

CONTENTS

Het immuunapparaat is een belangrijke factor in de bescherming van gewervelde dieren en de mens tegen infektieuze ziekteverwekkers zoals bacteriën en virussen. De bescherming ontstaat doordat een sub-populatie van de witte bloedcellen (lymfocyten) in staat is om lichaamsvreemde produkten (antigenen) op een specifieke wijze te herkennen als niet-zelf. Lymfocyten herkennen een antigeen via receptoren op hun celoppervlak. De receptoren binden dat gedeelte van het antigeen dat er ruimtelijk in past, analoog aan het sleutel-slot principe, en elke lymfocyt heeft één type receptor. Het aantal receptortypen wordt geschat op 10^9 per individu.

Het immuunapparaat biedt op twee manieren bescherming, op basis van twee subtypen lymfocyten: de B-cellen en de T-cellen.
De B-cellen zijn in staat tot de vorming van antistoffen. Antistoffen zijn eiwitten die in bloed en andere lichaamsvloeistoffen voorkomen. Zij hebben dezelfde receptor specificiteit als de B-cel die hen produceerde waardoor ze kunnen binden aan het antigeen. Binding van de antistof kan op verschillende wijzen resulteren in de inaktivering van het antigeen. Bacteriële toxinen en virussen worden belemmerd in hun interaktie met doelwitweefsels, en bacteriën kunnen worden gedood in samenwerking met het Complement systeem. Complement bestaat uit een serie eiwitten, die na aktivering door o.a. cel-gebonden antistoffen, een gat kunnen slaan in het celoppervlak hetgeen resulteert in celdood. Tevens mobiliseren de geaktiveerde complementfaktoren andere witte bloedcellen naar de infektieplaats. Deze cellen (macrofagen) kunnen het gedode materiaal opnemen en verteren.

T-cellen maken geen antistoffen, maar zij kunnen lichaamscellen die door een virus zijn geinfekteerd via cel-cel kontakt op specifieke wijze doden. Tevens produceren zij faktoren waardoor sommige bacteriën die in de macrofaag voortleven en delen, alsnog kunnen worden afgebroken. Naast deze effektorfunkties, hebben T-cellen ook een zeer belangrijke regelende taak. Via

helper, en suppressor T-cellen wordt de intensiteit van de immuunrespons van zowel B-cellen als van effektor T-cellen aangepast aan de aard en de intensiteit van het antigene kontakt.

Na een eerste kontakt met antigeen duurt het 1-2 weken voordat de effektorfunkties volledig zijn ontwikkeld. In deze primaire respons echter, heeft het immuunapparaat tevens de funktie ontwikkeld om bij volgende kontakten sneller en adequater te reageren. Dankzij deze geheugenfunktie kunnen mensen en dieren met succes worden gevaccineerd. Toediening van onschadelijk gemaakte, niet meer infektieuze bacteriën en virussen op jonge leeftijd induceert een primaire immuunrespons, en de daarbij ontwikkelde geheugenfunktie is zo afdoende dat latere infekties ongemerkt voorbijgaan; we zijn er immuun voor geworden.

Het onderscheid tussen zelf en niet-zelf, het vermogen om specifiek met een bepaald antigeen te reageren, en de geheugenfunktie daarin, zijn de fundamenten van het immuunapparaat.Dankzij deze eigenschappen zijn gewervelde dieren en de mens in staat tot een mobiel bestaan. Zij zijn gewapend tegen onbekende ziekteverwekkers in andere gebieden, en tegen veranderende omstandigheden in hun eigen woonomgeving. De herkenning van vreemd en het destructieve antwoord daarop houdt echter ook in, dat de uitwisseling van weefsels en organen tussen verschillende individuen niet mogelijk is, als de kenmerken op het weefsel (Grieks: histos) van de donor niet compatibel (verdraagzaam) zijn met die van de ontvanger. In dat geval worden de weefselkenmerken als histocompatibiliteitsantigenen herkend, en wordt het transplantaat afgestoten.

Van de verschillende histocompatibiliteitsantigenen die bekend zijn, is er één systeem dat door de sterkte van de respons die opgeroepen wordt speciaal opvalt: het "Major Histocompatibility Complex" (MHC). Omdat de MHC-antigenen bovendien worden gekenmerkt door een grote diversiteit, zijn niet-verwante individuen binnen een soort waarlijk uniek. Dit betekent dat de verschillen tussen donor en ontvanger teruggebracht kunnen worden

door de best passende combinatie te selekteren, maar dat de overeenkomst slechts volledig is bij een een-eiïge tweeling. Teneinde afstoting te voorkomen moet daarom het immuunapparaat van de ontvanger worden onderdrukt.

De middelen die voor deze immunosuppressie ter beschikking staan zijn niet in alle gevallen even effektief en bovendien werken ze op een niet-specifieke wijze. Het totale immuunapparaat wordt onderdrukt, en daardoor bestaat er een verhoogde kans op infektieziekten en tumoren. Vanuit dat oogpunt wordt grote aandacht besteed aan de ontwikkeling van andere therapieën. Op het laboratorium van de afdeling Nierziekten wordt onderzoek verricht naar de toepassingsmogelijkheden van zulke alternatieve therapieën. Gezien het experimentele karakter daarvan, wordt het onderzoek gedaan in een diermodel, waarbij huidtransplantaties worden verricht op muizen. In dit proefschrift zijn studies beschreven die gericht waren op het verkrijgen van een beter inzicht in het werkingsmechanisme van een pharmacologische en van een immunologische wijze om de transplantaatafstoting te onderdrukken.

CHAPTER 1

Although the use of donor tissue to support life for others has been imagined since long (1), transplantation of organs and tissues on a large scale is a relatively new development in medicine. Following pioneering work in the late 1950s (2-5), kidney transplant programmes were initiated at various centers and a total of over 70.000 kidneys have been grafted today (6, 7). In a recent survey of the results of 354 kidney transplantations in Nijmegen over the period between 1968 and 1981, it was reported that 70% of these grafts were functioning at one year, dropping to 56% at 5 years (8). Patient survival was 86% and 76% respectively. When these figures are applied to the world-wide scale, it follows that 21.000 kidneys were lost one year after transplantation and this is most likely a gross underestimation. Therefore, the situation is far from ideal and graft rejection remains the major problem to overcome. This applies even more stringently for organs such as heart and liver, because there is no long term artificial substitute for their vital functions comparable to the possibility of a return to haemodialysis in case of kidney graft failure.

Rejection of organs and tissue grafts is caused by differences in tissue antigens between donor and recipient. These so-called histocompatibility antigens are encoded by several distinct genetic systems, but one Major Histocompatibility Complex (MHC) has been identified, in all species analyzed (9). Studies in man (10) and in the mouse in particular (11), have revealed that the MHC-encoded antigens induce the most rapid graft rejection and that the system itself is characterized by an extreme polymorphism and complexity (12, 13). These phenomena have established a unique position for the MHC, and have set it apart from the other so-called minor histocompatibility antigens.However, due to the polymorphism of the MHC, unrelated individuals are truly unique, and despite matching procedures for the most prominent antigenic differences, transplantation of a cadaveric kidney is far less efficient than a kidney graft from a living

relative (8).

The immunological basis of graft rejection was first shown by Medawar in 1944 using skin grafts in rabbits (14) and in subsequent years, both cellular (15) and humoral (16) mechanisms were identified. To overcome rejection, pharmacological agents are used that depress the immune system. However, from the graft survival rates it is clear that this is not always realized. Moreover, besides the toxic side effects of these drugs, their action on the immune system is non-specific, depressing also the response to bacteria and viruses. Therefore, more efficient agents, that would in addition specifically suppress the response to only the graft antigens, would be quite advantageous. Such drugs, however, have not been developed as yet. Nevertheless, progress has been made in the identification of non-specific immunosuppressive agents, that are more effective, and that may lack the toxic side effects of current therapy. The use of antibodies against human lymphocytes to treat rejection crises, which has been shown to be at least as effective as conventional steroid therapy, but much less dangerous (17), may be applied even more successfully, when appropriate monoclonal antibodies can be raised. In addition, the use of Cyclosporin A, a newly discovered fungal metabolite with profound immunosuppressive activity (18), has great therapeutic potential, especially now the molecule has been synthesized and analogous products can be prepared and tested (19).

As an alternative to pharmacological agents, an attempt to specifically suppress the recipients response to donor graft antigens may be provided by the immune system itself. Reminiscent of the feedback inhibition of enzymes by their products, antibodies have been shown to exert a suppressive influence on their own production (20). This effect is specific. It concerns only the response to the immunizing antigen and does not affect the remainder of the immune potential of the host. Suppression by antibody applies also to the cellular immune response, and under certain conditions, administration of antibodies to the

graft antigens of the donor may result in prolonged graft survival. This phenomenon, which was first described in the 1900s (21) has been called Enhancement (22). Its application, however, has been confined to experimental systems in animals, because first of all, enhancement is only effective in those selected combinations, in which the genetic disparity of donor and recipient is limited. Secondly, the risk of inducing antibody-mediated graft rejection rather than enhancement, has certainly prevented its clinical use, and this stresses our ignorance of the mechanisms underlying these phenomena.
Therefore, experimental work in animals has continued with as its ultimate goal, specific immunosuppression in clinical medicine, but for the short term, to gain insight into the fundamental regulatory processes which govern the immune response.

The studies described in this thesis were designed to elucidate mechanisms in the induction of unresponsiveness to skin allografts in the mouse. They focus primarily on the mechanism of immunological enhancement, with in addition, an investigation on the immunosuppressive effects of Cyclosporin A.

HISTOCOMPATIBILITY 1982

Although originally discovered because of its role in graft rejection, the biological significance of the Major Histocompatibility Complex of various species analyzed, greatly exceeds this trait. The MHC of the mouse, the H-2 system, controls some 60 biological phenomena (23) and undoubtedly, influences on immune responsiveness are the most fascinating traits. From studies on the latter phenomenon, a picture is now emerging which shows that the regulation of cell-cell interactions is the ancestral function of the MHC, with in higher vertebrates in addition, the guidance of the cellular immune response in its function of distinguishing self from non-self on cell surfaces. These aspects have been described extensively (24-29), and need not be discussed in detail here. Excellent reviews are also a-

vailable on basic immunogenetics (13,30-32) and therefore, only a brief outline of the H-2 complex and its significance will be given below.

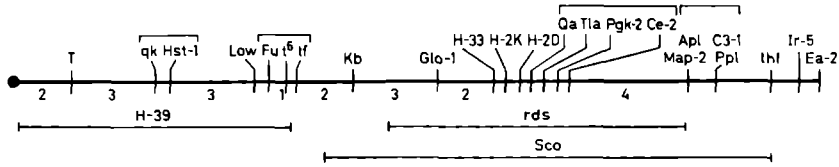

Figure 1. Genetic map of chromosome 17. The loci, from left to right are: centromere (●), brachyury (*T*), quaking (q^k), hybrid sterility-1 (*Hst-1*), low (*Low*), fused (*Fu*), anury (t^6), tufted (*tf*), histocompatibility-39 (*H-39*), knobbly (*Kb*), kidney catalase (*Ce-2*), histocompatibility-33 (*H-33*), histocompatibility-2K (*H-2K*), histocompatibility-2D (*H-2D*), Q antigen (*Qa*), thymus-leukemia antigen (*Tla*), phosphoglycerate kinase-2 (*Pgk-2*), retinal degeneration-slow (*rds*), plasma protein (*Plp*), complement 3-1 (*C3-1*), scopolamine modification of exploratory activity (*Sco*), acid phosphatase-liver (*Apl*), glyoxylase-1 (*Glo-1*), α-mannosidase processing 2 (*Map-2*), thin fur (*thf*), immune response-5 (*Ir-5*), erythrocyte antigen-2 (*Ea-2*). Brackets indicate that the order of loci within the bracket is unknown; segments indicate that the locus has not been mapped precisely and can lie anywhere within the limits of the segment. (Adapted from J. Klein (69)).

The H-2 complex is located on chromosome 17, as is shown in Fig.1. The genetic map of the H-2 complex itself is a matter for debate. Traditionally, the H-2 system is viewed as a chromosomal segment divided into four regions, K, I, S, and D/L and the I-region further divided into five subregions, I-A,I-B,I-J, I-E, and I-C (Fig.2). These divisions were made because various traits could be separated by crossing over. However, instead of calling an individual segment a locus, which is defined as a segment of genetic material coding for a single polypeptide chain, the terms region and subregion were introduced, because several traits could not be separated. They mapped to the same chromosomal segment, and because it was unknown whether these traits were controlled by the same locus or not, each trait was assigned a different locus symbol (33). Consequently, each region or subregion was initially defined by one locus, but with an increasing number of traits discovered, many more loci were assigned to them. Thus, each (sub)region controls several traits,

and each trait is presumably controlled by a separate gene product. The traditional H-2 map is depicted in Fig.2, with a functional translation in Table I.

Complex	H-2							
Ends	K						D	
Regions	K	I					S	D/L	
Subregions		A	B	J	E	C		
Loci	H-2K	Ir-1A	Ir-1B	Ia-4	Ia-5	Ir-1C	Ss,Slp	H-2D	H-2L
		Ia-1	Ia-2			Ia-3		Lad-4	
		H-2A	Ir-LDH$_B$			H-2C			
	Lad-3	Lad-1				Lad-2			
		Ir-(H,G)	A--L						
		Ir-OA							
		Ir-OM							
		Ir-BGG							

Figure 2: The traditional genetic map of the H-2 complex (adapted from J. Klein (33))

However, this interpretation has been challenged recently by the notion that a single H-2 locus is pleiomorphic, in that it controls a variety of traits (34). Evidence for this view was first of all provided by the study of H-2 mutations, which showed that a single mutation in a H-2K or H-2D locus, affected all traits controlled by that region (23). The same was true when I-region mutations were studied (35). In another approach, the expression of serologically detectable I-E region molecules was shown to correlate with the generation of cytolytic T-cells, whereas with antibodies against the I-A and I-E region encoded products, immune responsiveness could be affected (36). On the basis of these and other experiments, a new version of the H-2 complex has been proposed (34). In essence, it is a highly conservative functional model of the H-2 complex, because only those loci have been included, of which the products are characterized biochemically and which fit the pre-

Table I. *Functional aspects of the traditional H-2 map*[a)]

Region	K	I					S	D,L
Subregion		I-A	I-B	I-J	I-E	I-C		
Trait (locus)								
Serologically detectable antigens (H-2, Ia, SS, Slp)	++++	++++	———	+	++	———	++++	++++
Rejection of allografts (H)	++++	+++	———	———	++	?	———	++++
Cell-mediated lympholysis (H)	++++	+++	———	———	+++	?	———	++++
Mixed lymphocyte reaction (Lad)	++	++++	———	+	++			
Control of immune response (Ir)	+	++++	++	———	+++	———	———	———
(Is)	———	++++	———	———	———	++++	———	———
T_s-cell marker	———	———	———	++++	———	———	———	———
Control of T-B cell collaboration	———	++++	?	?	+++	?	———	———
Restriction of T_c-cell specificity	++++	———	———	———	———	———	———	++++
Restriction of T_h-cell specificity	———	++++	?	?	+++	?	———	———
Control of complement activity	———	———	———	———	———	———	++++	———

a) adapted from J. Klein et al. (34)

sumed physiological function of the MHC.

The complex is divided into class I and class II loci. The class I loci, K, D, and L, code each for 45 kd glycoproteins, which are noncovalently associated with a 12 kd β-2 microglobulin chain (37). They are expressed on nearly all cells, and their function is to guide cytolytic T-lymphocytes (38). The class II loci, Aβ and Aα, and Eβ and Eα, each code for 28 kd and 32 kd monomers respectively, which associate to form AβAα and EβEα dimers (39). They are expressed almost exclusively on B-cells and antigen-presenting cells (APC), and their function is the guidance of regulatory T-lymphocytes.

The guidance of T-lymphocytes is effected by their recognition of antigen in the context of MHC-molecules, and the recognition of self-MHC may thus enable the identification of non-self. The extreme polymorphism of the class I and class II loci is than from a teleologic viewpoint a most appropriate phenomenon, because polymorphism, and thus different capabilities of immunological responsiveness between individuals, protects the species from infections agents resembling self. Thus, MHC-polymorphism ensures the survival of the species, at the sacrifice of the individual.

It is not clear how the associative recognition of antigen and MHC-molecules by T-lymphocytes leads those cells to respond or not (40-42). In one view, it was postulated that defective reactivity of T-helper cells was due to defects in the physical association of class II molecules with antigen (43). Thus, according to this hypothesis, unresponsiveness functioned at the level of the antigen-presenting cell (44). Recently, however, this view was challenged by the observation that APC'c from non-responder mice were perfectly able to present at least the antigen tested to allogeneic T-cells (45). This suggested that in the syngeneic situation unresponsiveness functioned at the level of the T-cells, and might be caused by deficiencies in their receptor repertoire. These deficiencies might result from

the thymic education of T-cells and, as one of several possibilities, the selection of receptor repertoires for non-self MHC. In that case, T-cells do not recognize self, but "altered-self", and antigens which resemble self-MHC cannot be seen. Alternatively, unresponsiveness at the T-cell level might also be explained by the operation of yet another MHC-restricted response, the functioning of T-suppressor cells. Which of these interpretations will prove to be correct awaits further experimentation, but the importance of the MHC for the regulation of the immune response is indisputable, and the unravelling of the delicate interplay of complex regulatory circuits must ultimately reveal the final aesthetic simplicity of the immune system.

The new version of the H-2 complex may be the H-2 proper, according to what the function of MHC-molecules is thought to be. However, it is not claimed to be the final version. Firstly, the Qa and Tla loci, which encode products that have been termed class IV (27), may be part of the complex because structurally, and perhaps also functionally, they resemble the class I loci (37). The same is true for new, recently identified K- and D-region encoded molecules (46). Secondly, unique I-region encoded products have been detected on mature functional T-cells (47), whereas I-J controlled determinants are expressed on suppressor T-cells and on factors derived from these lymphocytes (48). It may be speculated that whereas class I molecules guide cytolytic effector functions, and class II molecules initiate functions on the APC-T cell, and T-B cell level, these molecules (class V?) guide T-T interactions at the regulatory level. Ample evidence has also been produced for the existence of carbohydrate defined I-region associated determinants (49) but their significance for the H-2 related functions remains also to be established. The same holds for the S-region which controls complement components (class III). In addition to these serological findings, analysis at the DNA-level progresses rapidly, and the presence of several different genes in the D^d-region has been reported (50). Therefore, a less conserva-

tive new version of the mouse H-2 complex may be visualized as is shown in Fig. 3. The H-2 map thus remains "as unstable as always" (23), but this merely reflects the prime importance of the study of the MHC, as was emphasized in 1980 by the Nobel Prize award in Physiology and Medicine, to B. Benacerraf, J. Dausset and G.D. Snell, who, amongst others, pioneered in these investigations.

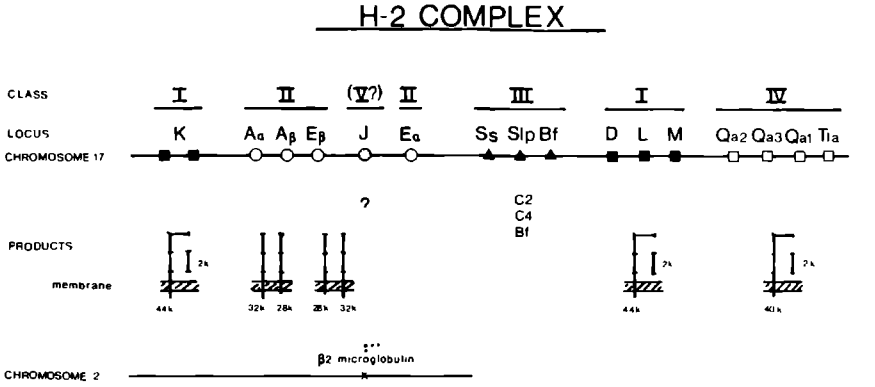

Figure 3. A new version of the H-2 complex (adapted from J. Klein (70))

The studies described in this thesis were performed mainly in a mouse skin allograft model, in which donor and recipients were inbred and genetically well defined. The H-2 haplotypes of these mouse strains are shown in Table II, and their genotypes in Table III.

Table II. *H-2 haplotypes of the mouse strains used*

Haplotype:	a	b	d	k
Strains:	A/HeJ	C57BL/6J	B10.D2/Sn-new	B10.BR
	B10.A	C57BL/10	B10.D2/Sn-old	BALB.K
		C57BL/Rɪj	BALB/c	C3H nu/nu
		C57BL/6 nu/nu	BALB/cByKh	
		B10.LP nu/nu	BALB/c nu/nu	
			BALB/cH-2^{dm2} a)	

a) BALB/cH-2^{dm2} is a mutant which does not express the H-$2L^{d}$ molecule

Table III. *H-2 genotypes of the mouse strains used*

strain			haplotype	K	Aα	Aβ	Eβ	J	Eα	S	D	L
A/HeJ	B10.A		a	k	k	k	k	k	k	d	d	d
C57BL	B10.LP		b	b	b	b	b	b	b	b	b	b
B10.D2	BALB/c		d	d	d	d	d	d	d	d	d	d
BALB/cH-2^{dm2}			d	d	d	d	d	d	d	d	d	-
B10.BR	BALB.K	C3H	k	k	k	k	k	k	k	k	k	k

ENHANCEMENT OF MOUSE SKIN ALLOGRAFTS

Enhancement may be viewed as an anomalous immunological phenomenon. The growth of an allograft in the presence of antibodies directed to the graft antigens is not inhibited, but favoured instead. This phenomenon, and the hypotheses which have been proposed to explain it have been excellently reviewed over the years (51-59) and therefore, only a frame will be pictured here.

First of all, understanding of the enhancing process was increased considerably when it was demonstrated for mouse skin allografts, that the protective effect of alloantibodies was changed into a destructive one, in the presence of a heterologous complement source (60). This suggested that the protective effects of alloantibodies could come to light because of an inefficiency of mouse complement to mediate acute rejection. This proved to be correct in subsequent studies (61). It also offered the possibility for the separate study of protective and destructive effects, in the same transplantation model. Passive enhancement could be induced by administration of alloantisera on days 0, 2, and 4 after grafting, whereas acute antibody-mediated rejection (AAR) was evoked by the same alloantibodies, but administered simultaneously with rabbit complement, on day 7 after grafting.

With these protocols, protective and destructive activities were distinguished following the separation of alloantibodies in different immunoglobulin subclasses, and according to their anti-class I and anti-class II specificities. The results can be summarized as follows:

1. AAR is mediated by complement fixing antibodies with specificity for class I antigens (62,63).
2. Enhancement is primarily mediated by alloantibodies with specificity for class II antigens (63), but also by anti-class I antibodies, although with the latter, the effects are small (64,65).
3. Both IgG2 and IgG1 antibodies are enhancing but on the basis of protein injected, IgG1 is less efficient than IgG2 (62).

Thus, these findings demonstrated that non-destructive enhancing antibody preparations should consist of either anti-class II sera, or of non-complement fixing IgG1 antibodies. Moreover, the apparent differences in the enhancing capacities of IgG1 and IgG2 suggested that the Fc-fragment of enhancing antibodies played a crucial role, and might be involved in their ultimate effect. This proved to be true, because F(ab')2 fragments were completely devoid of enhancing activities, as was demonstrated using skin (66) and tumor allografts (67).

The significance of the Fc-part of enhancing antibodies for the mechanism of enhancement formed the basis of most of the studies described in this thesis, as is further detailed below.

OBJECTIVES OF THIS STUDY

The findings that enhancement is Fc-dependent raised the question how this would be functionally linked to the induction of immune suppression. Since enhancement is specific, the interaction of enhancing antibodies with the graft antigens is clearly implicated and, as a consequence, antigen-antibody complexes might constitute the active principles in enhancement. Studies addressing this possibility are described in Chapter 2.

It was also asked how antigen-antibody complexes might exert their effect. As is shown in Fig.4 the possibility was considered that antigen-antibody complexes might be bound by specific antigen-reactive lymphocytes of the recipient, and that this would result in the inactivation of these cells, by a Fc-dependent mechanism. The Fc-dependent effector mechanisms responsible for inactivation might then be delineated using various immunoglobulin (sub)classes. In Chapter 3, studies are described in which the enhancing capacities of IgG and IgM antibodies were correlated with their opsonizing capacities.

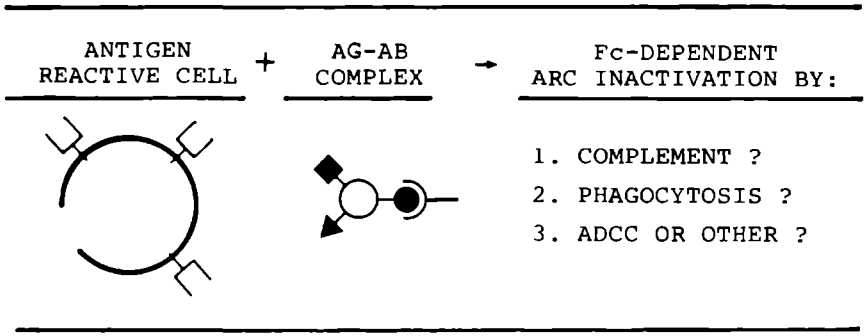

Figure 4. Hypothetical scheme for the inactivation of antigen-reactive cells (ARC) by antigen-antibody complexes, as a mechanism for immunological enhancement. Closed symbols represent the antigenic determinants on released graft antigen; ⟩— represents enhancing antibody; ADCC: antibody dependent cellular cytotoxicity

Since enhancement reflects the suppression of the cellular immune response, it was also asked whether the humoral response could be suppressed by alloantibodies in a similar Fc-dependent manner. For this purpose, the antibody response of nude mice to allogeneic and xenogeneic rat skin grafts was studied and the effects of IgG and IgM antibodies were analyzed, as is described in Chapter 4.

Studies on the effects of IgG1 and IgG2 alloantibodies in enhancement and opsonization are described in Chapter 5. In previous studies (62), IgG1 was shown to be less effective than

IgG2 in enhancement, but this comparison was based on the amounts of protein injected. Since IgG1 alloantibodies are not destructive because they cannot fix complement, it was important to re-establish their enhancing capacity, but this time on the basis of specific antibody activity. In addition, enhancement by IgG1 should give further information on the role of complement in the immune complex-mediated inactivation of antigen-reactive cells.

A different approach for the study of various immunoglobulin (sub)classes in enhancement is described in Chapter 6. Instead of tedious isolation procedures, the use of monoclonal antibodies offered the possibility to directly select for the relevant antibody (sub)classes, and their specificity. In addition, the use of monoclonal antibodies could provide valuable insight into the mechanism of enhancement; in order to interact with antigen-reactive cells, antigen-antibody complexes should display free antigenic determinants, the extent of which can be varied by using mixtures of various monoclonal antibodies.

Up till now, we have studied the induction of specific unresponsiveness by antibodies. However, an entirely different approach could not be neglected. As mentioned in the introduction, the recently discovered fungal metabolite, Cyclosporin A (CyA), displays profound immunosuppressive activities. Most remarkable however, is its specificity for T-lymphocytes and initial studies in the rabbit showed that a short course of CyA, induced the long-term acceptance of kidney grafts (68). As the effects were specific, it was proposed that CyA acted by the elimination of T-lymphocytes, specifically activated by the graft antigens. Consequently, a short course of CyA might deplete specific lymphocyte clones, and newly differentiated stem cells could see the graft as self. This hypothesis was tested using mouse skin allografts, as is decribed in Chapter 7.

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CHAPTER 2

EFFECTS OF ALLOANTIBODY, DONOR ANTIGEN, AND ANTIGEN-ANTIBODY COMPLEXES ON THE SURVIVAL OF MOUSE SKIN ALLOGRAFTS

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SUMMARY

Enhancement of mouse skin grafts by alloantibodies was compared with the effects of donor antigen and antigen-antibody complexes. Spleen cell membranes and detergent extracts thereof were used as sources of alloantigen. These preparations inhibited the lymphocytotoxicity of alloantibodies *in vitro*, and, depending on the route of administration and their form, they specifically primed prospective skin graft recipients for a secondary response, but did not induce active enhancement. Passive enhancement was readily demonstrated by the administration of alloantibodies at the time of grafting. However, after their incubation with alloantigen *in vitro*, to form antigen-antibody complexes, the enhancing effects of antibody alone were antagonized and not augmented. The same held true when, by the separate administration of alloantigen and alloantibody putative complexes were allowed to be formed *in vivo*. These results indicate that for the enhancement of mouse skin allografts, the use of alloantibody alone is superior to the administration of antigen-antibody complexes or donor antigen.

Pretreatment of prospective tumor graft recipients with donor antigen may result in prolonged graft survival, and anti-donor antibodies have been identified as one of the active principles (1-3). This phenomenon of Immunological Enhancement, which extends to normal tissues as well, can also be induced by the passive administration of alloantibody. The latter procedure is more reliable because active immunization may easily lead to accelerated graft rejection, due to the induction of cell-mediated immunity.

The role of antibodies in this type of immunoregulation may be explained by the assumption that immune complexes of anti-donor antibody and graft antigens interfere with immunocompetent cells of the host. Evidence for this originates from tumor systems, in which blocking of cell-mediated cytotoxicity by complexes *in vitro* was demonstrated (4). Immunosuppressive effects of antigen-antibody complexes have also been demonstrated in organ transplantation *in vivo*. Indirect evidence stems from the observations that the combined treatment with donor antigen and anti-donor antibody is more effective than the administration of either component alone (5,6), whereas similar effects have been reported with preformed complexes using liver grafts in baboons (7), and heart allografts in the rat (8,9).

If enhancing antibody acts by means of complexing with donor antigen *in vivo*, it might be expected that the size and the composition of these complexes varies with time, and that this influences their effectiveness. Thus, treatment with stable preformed antigen-antibody complexes might be superior to the administration of enhancing antibody alone. Therefore, we have investigated the effect of antigen-antibody complexes, in comparison with antibody or antigen alone. The results indicate that for the enhancement of mouse skin allografts, the use of alloantibody is superior to either donor antigen, or antigen-antibody complexes.

Breeding pairs of B10.D2/Sn-new (H-2^d), B10.BR (H-2^k), A/HeJ and B10.A (H-2^a), and C57BL/10 and C57BL/6J (H-2^b) were originally obtained from the Jackson Laboratory, Bar Harbor, Me. C57BL/Rij (H-2^b) originated from the Radiobiological Institute TNO, Rijswijk, The Netherlands. (C57BL/Rij x A/HeJ)F1 = B6AF1 and (C57BL/6J x A/HeJ)F1 = B6JAF1 were raised in the laboratory.

Alloantisera were prepared by weekly i.p. injections with a suspension of 5 x 10^7 lymphoid cells in Complete Freund's Adjuvant (CFA), as described earlier (10). Pooled ascites was heat-inactivated (56°C, 30 min), precipitated with (NH4)2SO4 and after solubilization sterilized by passage through membrane filters (Schleicher and Schüll, Dassel, West Germany) with decreasing pore sizes ranging from 8 µ to 0.2 µ. Rabbit anti-mouse lymphocyte serum (RAMLS) was prepared as described previously (10).

Spleen cell membranes were prepared essentially as described by Shimida and Nathenson (11), and solubilized with sodium deoxycholate (DOC) according to Cresswell (12). DOC-extracts were dialyzed extensively against buffered saline to remove detergent, and stored at -80°C, like the membranes. Total protein was determined by the method of Lowry et al (13) using bovine serum albumin as a standard.

Antigenic activity was determined by inhibition of lymphocytotoxicity. In the case of B10.D2 alloantigens, 25 µl anti-B10.D2 ascites, diluted to lyse 80-90% of B10.D2 spleen cells, were preincubated (37°C, 30 min) with a 25 µl dilution series of alloantigen, and thereafter this mixture was incubated (37°C, 30 min) with 25 µl B10.D2 spleen cells (5 x 10^6/ml) and 25 µl diluted (1:3) rabbit serum as a complement source. Antigenic activity is expressed as Inhibition Units (I.U.). One I.U. is defined as the reciprocal titer of 25 µl of alloantigen.

Transplantation of tail skin was carried out by a modification of the "fitted graft" technique, as described earlier (14). Each experimental group consisted of at least 5 mice.

Effects of donor antigen. Membranes and detergent extracts thereof (DOC-extract) were used as sources of alloantigen. The results of a representative isolation, using 100 B10.D2 mice, are shown in Table I.

Table I. *Isolation of alloantigens*

Preparation	Total protein (mg)	Specific activity (units/mg)	Recovery (%) protein	Recovery (%) activity	Purification
spleen cells	660	315	100	100	1
membranes	210	610	32	62	1.9
DOC-extract	160	705	24	54	2.2
lectin eluate	15	1890	2	14	7

Membranes and DOC-extract displayed specific antigenic activity *in vitro*, as was measured by the inhibition of lymphocytotoxicity (Fig.1). DOC-extract could be further purified by affinity chromatography on a lentil-lectin column, as described by De Waal et al (15), but this was accompanied by a 4-fold loss of material (Table I). Moreover, extensive dialysis resulted in a gel-like preparation, most likely due to the loss of stabilizing protein, and therefore, only the membranes and DOC-extract were analyzed *in vivo*.

Pretreatment of B6AF1 or B10.A recipients with B10.D2 alloantigen had either no noticeable effect on the survival of B10.D2 skin, or it resulted in sensitization. These effects depended on the form of the antigen, the route, and the time of administration. Priming for a secondary response was most prominent, if 28 days before transplantation a first B10.D2 skin graft was applied (Table II). Control survival times of 10.9 days were curtailed to < 7 days, because after removal of the band-aid the majority of test grafts was not healed in. Irradiated (2000 rads)

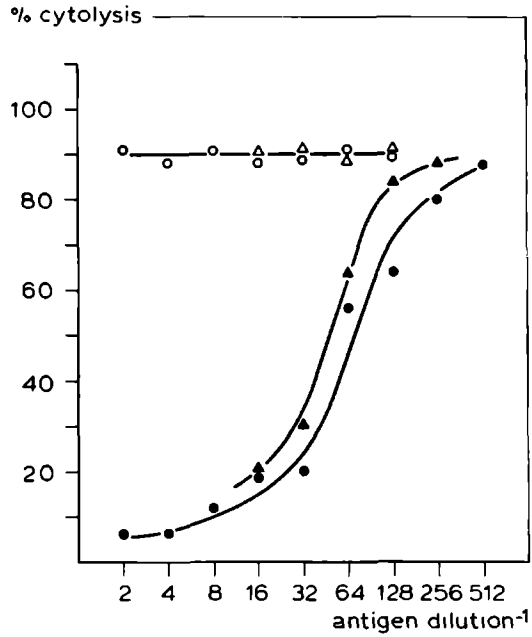

Figure 1. Specific inhibition of cytotoxicity by B10.D2 membranes (●-●) and B10.D2 DOC-extract (▲-▲). Inhibition was tested using diluted (1:300) B6AF1 anti-B10.D2 ascites with B10.D2 spleen cells (closed symbols), and B10.D2 anti-C57BL/Rij (1:200) with C57BL/Rij spleen cells as specificity control (open symbols)

spleen cells induced similar sensitization, but by contrast, an equivalent antigenic amount of membranes or DOC-extract did not. Only emulsified in CFA, these preparations specifically primed the host, when they were administered 28 days before grafting (Table II). Membranes were also non-immunogenic when injected i.v. at the time of grafting, even in higher doses (not shown). However, after i.p. injection shortly before grafting, sensitization was apparant, but this effect trailed of when the interval between immunization and transplantation became larger (Table III). These effects were not seen with DOC-extracts, which were non-immunogenic at any time, whether injected without adjuvant i.p., or i.v. It is evident from these findings that antigen pretreatment never resulted in prolonged graft survival, due to active enhancement.

Table II. *Survival of B10.D2 skin grafted onto B10.A recipients after different forms and routes of donor antigen administration 28 days before grafting.*

	MST ± S.D. (days)[a]			
		route of administration		
		i.v.	i.p.	i.p. + CFA
None	10.9 ± 1.1			
B10.D2 skin graft	< 7			
B10.D2 spleen cells (2.10^7)		7.8 ± 1.1	6.8 ± 1.1	7.6 ± 1.1
B10.D2 membranes[b]		11.0 ± 1.2	11.6 ± 1.1	8.2 ± 1.1
B10.D2 DOC-extract[b]		10.1 ± 1.0	9.8 ± 1.1	8.0 ± 1.0

a) Median Survival Time ± Standard Deviation (applies also to Tables III-IX)

b) 2 x 10^7 B10.D2 spleen cell equivalents

Table III. *Effects of i.p. injected B10.D2 membranes on the survival of B10.D2 skin grafted onto B10.A recipients. Influence of dose and time interval between immunization and grafting.*

Treatment	MST ± S.D. (days)			
	-7, -5, -3	p[a]	-17, -15, -13	p[a]
None	10.9 ± 1.1		10.9 + 1.1	
Membranes i.p.[b]				
5 µl	8.1 ± 1.1	< 0.005	9.9 ± 1.2	N.S.[c]
25 µl	7.0 ± 1.2	< 0.005	10.5 ± 1.1	N.S.
125 µl	7.0 ± 1.2	< 0.005	9.5 ± 1.2	N.S.

a) Level of significance (Student's t-test) in comparison with untreated controls

b) 1 µl is equivalent to 10^6 spleen cells

c) N.S., not significant

Effects of alloantibody. The efficiency of B6AF1 anti-B10.D2 alloantibodies was analyzed by induction of passive enhancement of B10.D2 skin grafted onto B10.A or B6AF1 recipients. As is demonstrated in Table IV, i.p. treatment of B6AF1 recipients on day 0, 2, and 4 after grafting resulted in a dose-dependent prolongation of graft survival from 10.9 till 19.9 days. However, when alloantibody was injected before grafting, little or no enhancement could be demonstrated. Using B10.A recipients, identical results were obtained (data not shown).

Table IV. *Passive enhancement by B6AF1 anti-B10.D2 alloantibody in B6AF1 recipients of B10.D2 skin grafts. Influence of dose and time interval*

Treatment	MST ± S.D. (days)		
	0, 2, 4	-7, -5, -3	-14, -12, -10
None	10.9 ± 1.1		
B6AF1 anti-B10.D2 serum			
1 µl	12.6 ± 1.2		
2 µl	15.5 ± 1.1		
5 µl	17.3 ± 1.1	13.2 ± 1.0	
15 µl	18.1 ± 1.1		
50 µl	19.9 ± 1.2	13.6 ± 1.1	12.2 ± 1.0

Effects of antigen-antibody complexes. On the basis of the results obtained with antigen or antibody alone, the effect of complexes was analyzed in several ways. Since antibody alone induced significant enhancement, it was analyzed whether complex-formation could augment this effect by using limited amounts of alloantibody and a non-immunogenic route of antigen administration. Using different amounts of membranes and two doses of alloantibody to arrive at varying ag:ab ratio's, we could demonstrate that complex formation did not augment enhancement, but antagonized it (Table V).

Table V. *Effect of preformed immune complexes injected i.v. on days 0, 2, and 4 after grafting of B10.D2 skin onto B6AF1 recipients*

Treatment	Ag:Ab ratio	MST ± S.D. (days)
2 µl anti-B10.D2		13.4 ± 1.0
+ 0,2 µl membranes	1 : 10	12.2 ± 1.0
+ 2 µl "	1 : 1	12.4 ± 1.2
+ 20 µl "	10 : 1	12.2 ± 1.2
25 µl anti-B10.D2		17.3 ± 1.1
+ 2 µl membranes	1 : 10	14.5 ± 1.1
+ 25 µl "	1 : 1	12.8 ± 1.1
+ 125 µl "	5 : 1	12.0 ± 1.2

In a second protocol use was made of the finding that antibodies administered before grafting did not induce enhancement, whereas DOC-extract injected i.p. was non-immunogenic. However, as is shown in Table VI, graft survival was not prolonged significantly. If instead of DOC-extract, membranes were used under these conditions, sensitization resulted at an ag:ab ratio of 5:1 (Table VI), confirming the results depicted in Table III.

Negative results were also obtained in a different donor-recipient combination, B10.BR→B10.A, using complexed B10.BR DOC-extract injected i.v. 17 days before skin grafting (Table VII). Since a combination of antigen-pretreatment with anti-lymphocyte serum was reported to be highly effective in the prolongation of skin graft survival (16), the effects of antigen or complexes were also analyzed in immunosuppressed B10.A recipients, but these results were also negative (Table VII).

In another approach, B10.D2 antigen and alloantibody were injected separately, in an attempt to induce complex formation *in vivo*. It was reasoned that the injection of B10.D2 DOC-extract intracutaneously as a depot in Incomplete Freund's Adjuvant

Table VI. *Effect of preformed immune complexes injected i.p. on days -7, -5, -3 on the survival of B10.D2 skin grafted onto B6AF1 recipients*

Treatment	Ag:Ab ratio	MST ± S.D. (days)
1 µl anti B10.D2		10.9 ± 1.1
+ 15 µl membrane extract	15 : 1	11.2 ± 1.1
+ 30 µl " "	30 : 1	12.2 ± 1.1
+ 60 µl " "	60 : 1	12.6 ± 1.1
+ 150 µl " "	150 : 1	10.7 ± 1.1
+ 1 µl membranes	1 : 1	10.2 ± 1.1
+ 5 µl "	5 : 1	7.6 ± 1.1

Table VII. *Effect of the i.v. administration of antigen and antigen-antibody complexes in normal, and in immunosuppressed female B10.A recipients of B10.BR skin*

Treatment	Ag:Ab ratio	MST ± S.D.		p[b]
		- RAMLS	+ RAMLS[a]	
None		11.2 ± 1.1	45.2 ± 1.4	
B10.BR DOC extract[c]		11.9 ± 1.2	37.3 ± 1.2	N.S.[d]
+ anti B10.BR serum	1 : 1	11.1 ± 1.1	36.6 ± 1.2	N.S.[d]

a) Rabbit anti-mouse lymphocyte serum (0.25 ml) was administered on days 0, 2, and 4 after grafting

b) Level of significance (student's t-test) in comparison with RAMLS treated controls

c) 160 µl B10.BR DOC-extract was injected i.v. 17 days before grafting

d) N.S., not significant.

might be comparable to a skin graft. However, using C57BL/10 recipients in these experiments, higher doses of antigen cur-

tailed graft survival, whereas the lowest amount tested (15 µl) induced weaker effects than enhancing antibody alone, given at the time of grafting (Table VIII). Also with lymphocytes injected i.v. 11 days, and alloantibody i.v., 10 days before grafting, a protocol efficient in the enhancement of rat renal allografts (6), sensitization was apparent using higher doses, whereas with lower amounts of cells, no enhancement could be induced (Table VIII).

Finally, the effect of established enhancing conditions on the survival of a second skin graft was evaluated, in order to gain insight into the dynamics of the enhancing process. Enhancing conditions were induced by the administration of alloantibody on days -10, -8, and -6 to B6JAF1 recipients which were grafted with B10.D2 skin on day -10. In order to manipulate the antigenic challenge, the residence of these first grafts was varied. They were removed either at day -8, -6, or -3. The effect of these protocols was analyzed by regrafting the recipients with B10.D2 skin on day 0. As is shown in Table IX, group 4, pregrafting in the absence of antibody resulted in sensitization, the magnitude of which was dependent on the time that these first grafts resided on their hosts. This sensitizing effect could be modulated by administration of alloantibody on days 0, 2, and 4, but only if the residence of these first grafts did not exceed 3-4 days (group 5). In these groups, where first grafts were removed at days -8, or -6, the sensitizing effect could be prevented completely by the administration of alloantibody at the time of first grafting (group 6), and the survival of second grafts was prolonged in comparison with untreated controls (group 1). However, the prolongation was not greater than graft survival in those recipients which had received antibody alone (group 3). If in addition, antibody was also administered on days 0, 2, and 4 (group 7), graft survival improved again but it was inferior to the standard treatment for the induction of enhancement, the administration of alloantibody on days 0, 2, and 4 after grafting (group 2).

Table VIII. *Effect of the separate administration of alloantigen and alloantibody in C57BL/10 recipients of B10.D2 skin*

Group	Treatment		Timing (days)	Route[a)]	MST ± S.D.	
					Controls	Complex treated
1	none				11.3 ± 1.1	
2	alloantibody[b)]		0, 2, 4	i.p.	16.6 ± 1.1	
3	alloantibody		-7, -5, -3	i.p.	11.2 ± 1.1	
	+ DOC-extract[c)]	- 15 µl	-7	i.c.		13.1 ± 1.1
		- 50 µl	"	"		8.5 ± 1.1
		- 150 µl	"	"		9.7 ± 1.1
4	alloantibody		-10	i.v.	12.0 ± 1.3	
	+ lymphocytes	- 10^4	-11	i.v.		10.9 ± 1.1
		- 10^5	"	"		8.9 ± 1.2
		- 10^6	"	"		8.1 ± 1.0
		- 10^7	"	"		7.0 ± 1.0

a) i.p.: intraperitoneally, i.v.: intravenously, i.c.: intracutaneously

b) alloantibody was always administered in doses of 0.25 ml

c) DOC-extract was emulsified in Incomplete Freund's Adjuvant

Table IX. *The influence of a first graft applied on day -10 on the survival of a second graft applied on day 0, and the effects of enhancing alloantibody*

Group	Treatment with antibody on days		MST ± S.D. (days)			
	-10, -8, -6	0, 2, 4	no 1st graft	1st graft removed at day		
				-8	-6	-3
1	-	-	10.9 ± 1.1			
2	-	+	17.5 ± 1.1			
3	+	-	13.6 ± 1.0			
4	-	-		7.6 ± 1.1	7.1 ± 1.1	<7[a)]
5	-	+		10.8 ± 1.3	9.9 ± 1.3	<7[a)]
6	+	-		14.1 ± 1.1	13.0 ± 1.1	9.6 ± 1.1
7	+	+		16.2 ± 1.1	16.4 ± 1.1	9.7 ± 1.2

a) Second grafts were not healed in

The results decribed in this paper demonstrate that all protocols which were designed to analyze the role of antigen-antibody complexes in enhancement, were inferior to the standard regimen, the administration of alloantibodies at the time of grafting. The possibility that the negative results with preformed complexes were due to the loss of H-2 antigens during isolation was excluded, because the antigeniticy of membranes and detergent extract thereof, was demonstrated by inhibition of lymphocytotoxicity. Moreover, their immunogenicity was apparent from the sensitizing effects in prospective skin graft recipients. It is also unlikely that preformed complexes were inadequate due to their composition, because various ag:ab ratio's were analyzed. However, it cannot be excluded that due to their size, which was not investigated, preformed complexes were readily removed from the circulation by phagocytosis. This may particularly apply for complexed membranes. As a consequence, antibody within such a complex would be removed as well, and this may explain why complexed membranes injected i.v.,were less efficient than the corresponding amount of antibody alone.

Therefore, it was analyzed whether the separate administration of antigen and antibody, and putative complex formation *in vivo*, could induce immunosuppression. Because antibody alone induced significant enhancement when injected at the time of grafting, pretreatment protocols were adopted in these experiments. The data demonstrate however, that both with DOC-extract, lymphocytes, and a skin graft as sources of antigen, the combined treatment was inferior to antibody alone.

Analysis of the survival of second grafts on recipients pretreated with a first graft revealed that this had resulted in senzitization. Nevertheless, administration of enhancing antibody during second grafting was able to modulate the destructive effects of the existing effector cells. This so-called efferent blockade was, among others, initially proposed to explain

enhancement (3). However, the protective effects reported here were only apparent if the antigenic challenge with the first graft was short. No effect of antibody was demonstrated if first grafts resided for longer than 4 days on their recipients. Similar results were recently obtained by others. Transfer of immune spleen cells to skin grafted mice resulted in accelerated rejection, and the administration of enhancing alloantibody could only partially abrogate this effect (17). As demonstrated here, and by others (18), enhancing antibody only suppresses the primary immune response and it is ineffective in sensitized recipients. This indicates that, although an interference with effector cells may occur to some extend, it cannot fully explain enhancement.

Our data show that it is more likely that antibody interferes with the induction of immunity, because the sensitizing effect of first grafts could be abrogated by the simultaneous administration of alloantibody. The dose of antigen was clearly important, because with greater numbers of lymphocytes, or a longer residence of first grafts, sensitizing effects re-appeared. This explains, at least in part, some of the negative results with antigen-antibody complexes. On the other hand, in several experiments graft survival was not influenced at all, or equivalent to the effect of antibody alone. This may indicate that antibody merely acted by the removal of antigen and thereby reduced the antigenic stimulus (3, 19). This possibility cannot be excluded on the basis of our data, but evidence exists that also this mechanism cannot fully explain enhancement (20). The most striking evidence which argues against this mechanism, is the recent demonstration of tumor enhancement by anti-TNP antibody and TNP-conjugated alloantigen, because the anti-TNP antibody cannot interfere with the graft itself (12). Instead, this argues strongly for the role of antigen-antibody complexes, and it has been proposed that antigen-antibody complexes opsonize antigen-reactive cells, which may result in the elimination of these lymphocytes by phagocytosis (22). That in our hands, antigen-antibody complexes were ineffective, may be caused by the

pretreatment protocols that were used. It has been demonstrated in the enhancement of rat kidneys (23-25), and mouse skin (26, 27), that enhancing antibody induced a delay in the induction of cell-mediated immunity, but once re-appeared, that the strength of the response is not decreased. This suggests that the effect of antigen-antibody complexes on antigen-reactive cells occurs locally and may be short lasting. Opsonization may result in a reduction of peripheral lymphocytes, but due to the recruitment of cells from secondary lymphoid organs, this effect may only be temporal. This may explain why pretreatment was ineffective, whereas, as reported by others, the use of antigen-antibody complexes at the time of grafting induced significant enhancement (7-9,21).

Our results with antigen pretreatment showed that the immunogenicity of MHC-antigens depended on their form, the route of administration, and the timing, and this is in agreement with results from others (28). Nevertheless, administration of donor antigen resulted in accelerated rejection, rather than enhancement. Recently, the specific suppression of DTH to alloantigens was reported, using preimmunization with irradiated allogeneic cells (29). When we applied this system to induce suppression for grafted skin however, accelerated rejection resulted in all experiments (unpublished observations). Similarly, accelerated rejection of thyroid allografts has been reported in mice, that showed severely depressed MLC and CML activities following the injection of 10^8 donor spleen cells (10). However, that the principle of unresponsiveness induced by antigen pretreatment does apply to skin grafts was recently demonstrated in our laboratory, using BALB/c H-2^{dm2} recipients of BALB/c Bykh skin, pretreated with donor spleen cells (manuscript in preparation). As this combination only differs for the H-$2L^d$ antigen, these results indicate that the extend of the MHC-barrier may be critically involved.

In conclusion, we have demonstrated that the use of alloantibody alone, was superior to the use of donor antigen, or antigen-an-

tibody complexes for the enhancement of mouse skin allografts, and that enhancing antibody operates at the induction phase of the immune response. The negative results with antigen-antibody complexes may indicate that their effect, if any, is not long-lasting.

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CHAPTER 3

EFFECTS OF IgG AND IgM ALLOANTIBODIES IN THE ENHANCEMENT OF MOUSE SKIN ALLOGRAFTS AND THE RELATION WITH THEIR OPSONIZING CAPACITY IN VIVO

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EFFECTS OF IgG AND IgM ALLOANTIBODIES IN THE ENHANCEMENT OF MOUSE SKIN ALLOGRAFTS AND THE RELATION WITH THEIR OPSONIZING CAPACITY *IN VIVO*[1]

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The capacity of IgG and IgM alloantibodies to enhance the survival of B10.D2 skin, grafted onto B6AF$_1$ recipients, was compared with their opsonizing capacity *in vivo*.

IgM and IgG were purified by gel filtration on a Sephacryl S-300 column, using B6AF$_1$ anti-B10.D2 serum, which was collected 8 days after immunization. Administration of IgG together with rabbit C resulted in acute antibody-mediated graft rejection (AAR), whereas IgG alone was able to induce enhanced graft survival. By contrast, administration of IgM, with cytotoxic activity similar to IgG, and similar activity in AAR, did not result in enhancement.

Prior incubation of ^{51}Cr-labeled leukocytes with IgM failed to change their *in vivo* spleen/liver distribution, whereas with IgG antibodies a profound liver diversion was observed. These results show that enhancing IgG antibodies are opsonizing *in vivo*, whereas nonenhancing IgM antibodies are not. They support the recently proposed hypothesis that opsonization of antigen-reactive cells (ARCO) is involved in the induction of enhancement.

Passive administration of alloantibodies to recipients of an allograft can influence graft survival in several ways On the one hand, binding of antibodies to allografts may lead to hyperacute complement- (C) dependent graft rejection (1) In the mouse having an inefficient C system this phenomenon can only be demonstrated if an effective heterologous C source is administered simultaneously with alloantibody (2, 3) On the other hand, immune responsiveness to the graft antigens can be manipulated by alloantibodies, and the phenomenon of immunologic enhancement of allografts is an example of the suppression of the immune response (1, 4)

We have shown previously that enhancement is an Fc-dependent phenomenon Highly purified F(ab')$_2$ fragments of alloantibodies were unable to prolong skin allograft survival or to enhance tumor growth in the same mouse model (5, 6) As a first approach to the investigation of the nature of this Fc dependence, we have compared the effects of IgG with those of IgM alloantibodies The role of IgM in the induction of enhancement has been studied mainly in tumor models In various reports it was shown that IgM alloantibodies were able to induce enhanced tumor growth with either less efficiency than IgG (7) or with comparable effectiveness (8–10) However, using similar models, other authors were unable to detect enhancing effects mediated by IgM (11–15), and comparable failures were reported with kidney transplants in the rat (16–18) The controversy extends to systems where administration of IgG antibodies induces specific suppression of antibody formation, since here also ineffectiveness (19) as well as definite suppressive activity (20) of IgM was demonstrated

The uncertainty about the role of different immunoglobulin classes in the induction of antibody-mediated immune suppression is related to unanswered questions concerning the mechanism of this phenomenon Recently, it was proposed that the induction of enhancement might be the result of the phagocytosis of specific antigen-reactive cells, which had bound immune complexes consisting of released graft antigen and administered alloantibody (21) This phenomenon has been called antigen-reactive cell opsonization (ARCO) [3]

We have therefore compared the effects of IgG and IgM alloantibodies in the enhancement of skin allografts in relation to their opsonizing capacity in the same mouse model The induction of acute antibody-mediated rejection (AAR) by these antibodies was used as a probe for their *in vivo* reactivity It will be shown that IgM antibodies were neither enhancing nor able to opsonize, in contrast

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[3] Abbreviations used in this paper AAR acute antibody-mediated rejection ADCC antibody-dependent cell-mediated cytotoxicity ALS anti-lymphocyte serum, ARC antigen-reactive cells ARCO antigen-reactive cell opsonization CTU cytotoxic unit LI localization index B10AF$_1$ (A/HeJ × C57BL/10)F$_1$ B6AF$_1$ (A/HeJ × C57BL/6Rij)F$_1$

to IgG antibodies, which were active in enhancement as well as in opsonization

MATERIALS AND METHODS

Animals Inbred strains of B10 D2 old Sr B10 D2 new Sn B10 BR A/HeJ and C57BL/10 mice were originally obtained from The Jackson Laboratory (Bar Harbor ME) and C57BL/6Rij mice from the Radiobiological Institute Rijswijk The Netherlands B10AF and B6AF hybrids were matings of A/HeJ and C57BL/10 and of A/HeJ and C57BL/6Rij respectively B6AF hybrids were used as skin-graft recipients whereas B10AF mice were used in liver diversion experiments The non-H 2 differences (H 9) between C57BL/10 and C57BL/6 have no effect on immune responsiveness as measured by skin-graft rejection (22)

Antisera $B6AF_1$ anti-B10 D2 serum was obtained by an i p injection of a suspension of 5 × 10 B10 D2 lymphocytes in addition to injection of a similar number of lymphocytes emulsified in complete Freund s adjuvant (CFA Difco Detroit MI) Peak titers of IgM antibody activity were obtained 8 days after immunization as judged by sucrose density gradient centrifugation (23) and cytotoxicity assay Pooled sera were treated with $(NH_4)_2SO_4$ at 50% saturation and the precipitated proteins solubilized in PBS were sterilized by passage through a series of membrane filters (Schleicher and Schull Dassel West Germany) with decreasing pore sizes ranging from 8 μ to 0 2 μ

B6AF anti-B10 D2 ascites fluid was obtained by weekly i p injections of a suspension of 5 × 10 B10 D2 lymphocytes in CFA as described earlier (5) Anti-lymphocyte serum (ALS) was raised in goats by a subcutaneous injection of 5 × 10^8 C57BL 6Rij lymphocytes suspended in CFA followed by 2 weekly i v injections of 5 × 10^8 C57BL/6Rij lymphocytes in saline Anti-mouse erythrocyte antibodies were removed from the ALS by adsorption with C57BL/6Rij erythrocytes Antisera used *in vivo* were heat-inactivated at 56°C for 30 min

Isolation of IgM and IgG Separation of IgM from IgG was achieved using gel filtration of 4-ml aliquots on a 3 x 100 cm column containing Sephacryl S-300 superfine (Pharmacia Uppsala Sweden) Eluted fractions were analyzed for IgM IgA and IgG by means of rocket electrophoresis against class-specific anti-mouse antisera (Meloy Labs Springfield VA) IgM- and IgG-containing fractions were pooled and concentrated to their original volumes using YM-10 and XM 50 filters respectively (Amicon Lexington MA) Cross-contamination was assessed by radial immunodiffusion and the purified IgM fractions were rechromatographed at least once on the same column

Cytotoxicity assay The *in vitro* cytotoxic activity of alloantisera was determined by trypan blue exclusion with B10 D2 B10 BR or B10AF spleen cells freed of erythrocytes by treatment with NH_4Cl Using microliter plates (Greiner Nurtingen West Germany) 25 μl of a cell suspension (5 × 10^6/ml) were incubated with 25 μl of a dilution series of alloantibody in Hanks balanced salt solution and 25 μl diluted (1 4) rabbit serum as the C source for 30 min at 37°C The rabbit sera were preselected and only those sera that lysed 10% or less of the mouse lymphocytes in the absence of alloantibody were used

Liver diversion of antibody coated spleen cells B10 D2 spleen cells (1 5 × 10^8 ml) freed of erythrocytes by treatment with NH_4Cl were incubated with 75 μCi of Na^5CrO_4 (The Radiochemical Centre Amersham U K spec act 100 to 350 μCi/μgCr) for 45 min at 37°C in Hanks balanced salt solution supplemented with 0 1% BSA After incubation the cells were washed twice in the cold and thereafter dead cells were removed as described by Hudson and Hay (24) Aliquots of 10^6 cells/0 1 ml were then incubated with varying concentrations of anti-B10 D2 antibody fractions for 30 min at room temperature washed once and injected i v into B10 D2 old or B10AF mice In addition to normal $B10AF_1$ recipients B10AF mice that had been grafted with B10 D2 skin 4 days earlier were used After 3 hr recipient mice were killed and livers and spleens were removed for gamma counting

In control experiments labeled B10AF spleen cells incubated with similar amounts of anti-B10 D2 antibody and also with an anti A HeJ serum as a positive control were used The presence of specific antibody on the cells to be injected was analyzed with the cytotoxic assay Samples were incubated with diluted (1 4) rabbit serum for 30 min at 37°C and lysis was estimated by trypan blue inclusion as well as by specific ^{5}Cr-release Doses of antibody are expressed in cytotoxic units (CTU) One CTU is defined as the amount of alloantibodies lysing 50% of 10^6 cells to be injected Results of the opsonization experiments are expressed as the percentage of injected counts present in the spleen and in the liver Localization indices (L I) were calculated by dividing the mean liver/spleen ratio of antibody-treated cells by the mean liver/spleen ratio of untreated cells as described by Hutchinson (21)

Skin grafting Transplantation of B10 D2 and B10 BR tail skin onto $B6AF_1$ recipients was carried out as described earlier (3)

Enhancement of allografts Recipients of B10 D2 and B10 BR skin grafts were injected i p with either 0 25 m serum or the IgM or IgG fractions thereof on days 0 2 and 4 after grafting

AAR Acute rejection of well-established grafts on immunosuppressed recipients was induced by i v injection of alloantibody together with 0 25 ml rabbit C at day 7 after grafting The recipients were immunosuppressed by i p injections of 0 25 ml ALS on days 0 2 and 4 after grafting The rabbit serum used as C source was preselected and had no cytotoxic effect on mouse lymphocytes *in vitro*

RESULTS

Purification of IgM and IgG The IgM fraction from anti-B10 D2 serum collected 8 days after immunization was prepared using salt precipitation and gel filtration Of the gel filtration media tested Sephacryl S-300 superfine gave the best results Initial attempts using Sephadex G-200 resulted in a suboptimal separation of IgM from IgA and IgG whereas the use of Bio-gel A 0 5 M resulted in considerable losses of IgM activity The loss of IgM activity was not due to its inherent instability as reported by others (13) Various treatments demonstrated that IgM was as stable as IgG (Table I)

However it has been reported that IgM interacts nonspecifically with lipoproteins that are adsorbed onto agarose matrices (25) Therefore we removed lipoprotein with $CaCl_2$/dextran sulfate (25) before gel filtration, but although this procedure improved the IgM recovery the results were still inferior to direct application of the Sephacryl column (Table I)

IgM containing fractions from a 1st run were concentrated to the original volume and rechromatographed once The 19S material from the 2nd runs was pooled and concentrated to roughly the same IgM concentration as was present in the original serum Overall IgM recovery was 53%, with one-fourth of the cytotoxicity of the original serum (Table II) Protein eluted in the 7S region of the first gel filtration runs was used as a source of IgG Pooled fractions were concentrated and characterized by radial immunodiffusion as shown in Table II Overall IgG recovery was 83%, with one-fourth of the cytotoxicity present in the original serum

AAR of skin allografts The *in vivo* antigen binding capacity of the various preparations was tested by their ability to induce AAR Graded amounts of antibody together with 0 25 ml rabbit C were administered i v to immunosuppressed B6AF recipients of B10 D2 skin on day 7 after grafting The results given in Table III show that similar amounts of IgG or IgM antibody, based on their lymphocytotoxic titers *in vitro*, were almost equally effective in the induction of complete necrosis of the grafts within 48 to 72 hr

Enhancement of skin allografts Having demonstrated the *in vivo* activity of the immune serum and of the purified fractions in AAR the ability to induce passive enhancement was tested The results shown in Table IV demonstrate that administration of early unfractionated anti-B10 D2 serum resulted in a significant prolongation of the survival of B10 D2 skin However this enhanced survival

TABLE I

Recovery of IgM antibody activity after various manipulations

Treatment	Recovery[a] (%)
Precipitation with $(NH_4)_2SO_4$	100
Incubation at 25°C (3 wk)	100
Freezing and thawing (6×)	100
Gel filtration on Bio-Gel A-0 5 M	25
Bio-Gel A-0 5 M after removal of lipoprotein	60
Gel filtration on Sephacryl S-300	75

[a] Lymphocytotoxic activity of the 19S fraction after sucrose density gradient centrifugation

TABLE II

In vitro properties of B6AF anti-B10 D2 alloantisera and of the IgG and IgM fractions

Preparation	Cytotoxic Titer	Concentration (mg/ml)[a]		
		IgM	IgG1	IgG2[b]
Hyperimmune ascites	1 1000	N D	5 05	4 11
Day 8 serum	1 128	0 72	2 71	2 35
IgM fraction	1 32	0 64	0 005	0 012
IgG fraction	1 32	<0 02[c]	3 82	4 60

[a] Determined by radial immunodiffusion
[b] IgG2a + IgG2b
[c] N D not done
[d] Less than the detection level of IgM

TABLE III

Activity of B6AF, anti-B10 D2 antiserum and the IgG and IgM preparation in acute antibody-mediated rejection in B6AF recipients of B10 D2 skin grafts (AAR)

Treatment[a]	Volume	AAR
	ml	
Day 8 serum	0 5	2/5[b]
	0 75	5/5
IgM fraction	0 125	0/5
	0 25	2/5
	0 5	5/5
IgG fraction	0 125	2/4
	0 25	4/5
	0 5	5/5

[a] AAR is evoked by administration of alloantibody together with 0 25 ml rabbit C to immunosuppressed recipients on day 7 after grafting

[b] Number of recipients showing complete necrosis of the graft within 48 to 72 hr divided by the total number injected

TABLE IV

Passive enhancement by B6AF, anti-B10 D2 alloantisera and the IgG and IgM preparations in B6AF, recipients of B10 D2 skin grafts

Treatment[a]	No Recipients	MST ± SD[b]	p[c]
None	10	10 3 ± 1 1	
Anti-CFA serum[d]	7	10 0 ± 1 1	N S[e]
B6AF, anti-B10 D2			
– Day 8 serum	9	14 5 ± 1 0	<0 0025
– IgG fraction	9	14 3 ± 1 1	<0 01
– IgM fraction	10	10 3 ± 1 1	N S
– Hyperimmune ascites	10	20 1 ± 1 1	<0 0005

[a] 0 25 ml administered on days 0, 2 and 4 after grafting

[b] Median survival time ± SD

[c] Level of significance (Student s *t*-test)

[d] Collected from B6AF, mice 8 days after injection of complete Freund s adjuvant

[e] Not significant

was caused exclusively by the IgG antibodies present in this serum IgM alloantibodies, effective in AAR, were completely devoid of enhancing activity, whereas the isolated IgG fraction demonstrated similar enhancement as the serum from which it originated The anti-B10 D2 serum and the IgG and IgM fractions thereof had no effect on the survival of B10 BR control skin grafts

Liver diversion of antibody-coated spleen cells According to the ARCO hypothesis, opsonization of antigen-reactive cells (ARC) is involved in the induction of enhancement Therefore, we analyzed whether the different effects of IgG and IgM in enhancement could be correlated with their opsonizing capacity *in vivo* Spleen cells of B10 D2 donor mice, labeled *in vitro* with ^{51}Cr, were incubated with anti-B10 D2 antibody and injected i v into recipient mice To exclude the possibility that C-induced lysis of these coated cells could be responsible for any observed liver diversion, the lytic activity of mouse C on these cells was investigated first IgG-coated spleen cells (10^5) were incubated *in vitro* with either 25 µl diluted (1 4) rabbit C or with 200 µl undiluted mouse C For this purpose, freshly drawn blood from male C57BL/10 mice was used, which has a relatively high lytic activity on antibody-coated SRBC (26) Using rabbit C complete cell lysis was observed, in contrast to mouse C, which had no lytic activity at all (data not shown) As a 2nd approach, spleen/liver localization of antibody-coated cells was investigated using congenitally C-5-deficient B10 D2 old mice

The results given in Figure 1 demonstrate the opsonizing capacity of IgG antibodies in the absence of lytic C activity Using suspensions of viable cells without prior incubation with antibody, the percentage of cells moving to the spleen exceeded the number of cells sequestered in the liver Cells incubated with IgG, however, were diverted away from the spleen, with a simultaneous increased localization in the liver in a dose-dependent manner

The opsonizing effect of IgG was then compared with IgM using B10AF, recipients In order to mimic the events occurring in the enhancement protocol as closely as possible, B10AF, mice were used, which had been grafted with B10 D2 skin 4 days before the diversion experiments, in addition to normal B10AF, recipients As a control, B10AF, spleen cells were used after incubation with the same amounts of anti-B10 D2 antibody and with an anti-A/HeJ serum The results in grafted recipients, given in Figure 2, show the specific and profound opsonizing capacity of IgG antibodies Moreover, the magnitude of the observed liver diversion was similar to the effects seen in B10 D2 old recipients By contrast, IgM antibodies were ineffective at 2 5 and 12 5 CTU, whereas the liver diversion seen with the highest amount tested could not be discriminated from the nonspecific effect displayed on B10AF, control cells No differences were observed between normal and grafted recipients, and therefore only the results in the latter group are shown

When the samples used for injection were incubated with rabbit C, B10 D2 cells incubated with IgG or with IgM were lysed com-

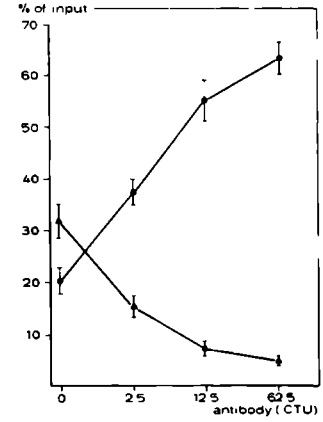

Figure 1 Dose-dependent liver diversion of B10 D2 splenic leukocytes coated *in vitro* with IgG alloantibody in B10 D2 old (C5-deficient) recipients The means ± SD of the counts recovered in spleen (▲——▲) and in liver (●— ●) of at least 3 mice per group are expressed as the percentage of the total dose injected

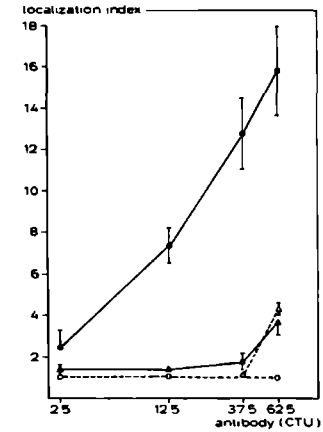

Figure 2 Localization indices in grafted B10AF, recipients of B10 D2 leukocytes incubated with anti-B10 D2 IgG (● ●) and IgM (▲——▲) alloantibody and of B10AF, leukocytes incubated with anti-B10 D2 IgG (○—○) and IgM (△—△) Each point represents the means ± SD of at least 3 recipients in 2 separate experiments

pletely, indicating the successful coating of these cells. B10AF$_1$ control cells that had been incubated with anti-B10 D2 IgG or IgM were not lysed under these conditions. Only after incubation of these cells with the relevant anti-A/HeJ serum could strong liver diversion and lysis with rabbit C *in vitro* be demonstrated.

DISCUSSION

In earlier studies from our laboratory, the enhancing capacity of IgG alloantibodies was established (27). Dose-response curves indicated that 27 μg IgG2 with a cytotoxic titer of 1:512 were able to induce significant prolongation of skin graft survival in the mouse model used. Similar studies demonstrated that IgG1 alloantibodies were also enhancing, although less effective than IgG2.

The enhancing capacity of IgG antibodies is confirmed in this study. By contrast, IgM antibodies failed to induce enhanced graft survival. To exclude that this result was due to inefficient antigen binding, the activity of IgM was tested *in vitro* and *in vivo*. Antigen binding and activation of rabbit C *in vitro* was demonstrated by lymphocytotoxicity. Interaction with the graft antigens *in vivo* was shown by the observation that, in terms of cytotoxic units, the IgM preparation was almost as effective in the induction of AAR as the IgG preparation. The activity of IgM in AAR cannot be the result of contaminating IgG. The IgM preparation contained 12 μg/ml of IgG2 and 5 μg/ml of IgG1. In the induction of AAR, using 0.5 ml, these contaminations amounted to 6 μg IgG2 and 2.5 μg IgG1, whereas the threshold dose of IgG2 to induce AAR is 40 μg, and IgG1 is ineffective in AAR in doses up to 2000 μg (27).

For the study of the opsonizing capacity of the IgG and IgM antibodies, a model was chosen consisting of the *in vivo* liver diversion, in normal and in grafted recipients, of lymphocytes labeled with ^{51}Cr and coated with antibody *in vitro*. By using an *in vitro* procedure, we were able to determine by means of a lymphocytotoxic assay whether the lymphocytes were indeed coated with antibody before their injection. Moreover, amounts of antibody could be used exceeding those attainable by administration *in vivo*.

In addition to normal recipients, grafted animals were used, in order to study opsonization in the natural physiologic environment in which enhancement takes place. No differences were observed between grafted and normal recipients, and the results demonstrate that in contrast to IgG, IgM has no opsonizing activity *in vivo*. The inability of IgM to induce enhancement is in agreement with results of others, using rat kidney allografts (16–18) and tumors (11–15), and it supports our findings that the antibody response of nude mice to allogeneic and xenogeneic skin grafts is abrogated by IgG, but not by 19S IgM antibodies (28). The results demonstrate also that for the production of enhancing antibodies, hyperimmunization is not a prerequisite, as suggested by Stuart *et al.* (29), but that the amount of specific IgG antibody present in the serum determines the enhancing capacity of early immune sera.

That IgM antibodies are not enhancing reinforces the concept that masking of antigenic determinants is not an adequate explanation for enhancement, since both nonenhancing IgM antibodies and enhancing IgG antibodies bind to the target antigens, as judged by their ability to induce AAR. The possibility that the IgG and IgM fractions nevertheless have different specificities is not very likely, because IgM and IgG were isolated during a primary immune response in which the specificities of IgM antibody precede those of IgG. That IgM antibodies are neither enhancing nor opsonizing extends previous findings that showed that enhancement is Fc-dependent (5, 6, 30).

It was recently proposed by Hutchinson (21) that passively administered antibodies could form immune complexes with cell bound or soluble antigens released from the graft. Recognition and binding by ARC, of these complexes in antigen excess could then result in ARC-inactivation by phagocytosis, as was demonstrated in rats bearing passively enhanced renal allografts (31, 32) and also in mice (33). Nevertheless, inactivation of ARC does not necessarily occur via opsonization alone, but other effector mechanisms like antibody-dependent cell-mediated cytotoxicity (ADCC) or C-mediated lysis could be involved. However, the liver diversion we observed in mice deficient in lytic C was of comparable magnitude in B10AF mice. Furthermore the fact that in mice AAR can only be induced after administration of heterologous C, and the observation that, in accordance with reports from others (34, 35), antibody-coated cells were not lysed by fresh mouse serum indicate that C-mediated lysis is not likely to occur *in vivo*.

Recent observations suggest that in mice IgM antibodies are able to mediate ADCC (36–39). If ADCC is involved in ARC inactivation, IgM antibodies should be able to induce enhancement by this route. Therefore the finding that IgM antibodies do not induce enhancement argues against ADCC as an effector mechanism for the inactivation of ARC.

From a teleologic point of view, it seems most rewarding that IgM antibodies do not abrogate immune responses. Instead, they could play a stimulatory role in the humoral response, as shown by Henry and Jerne (40) using antibody formation against SRBC in the mouse, as well as in the cellular immune response, as shown by Mullen *et al.* (16) using rat kidney allografts. These observations may be related to the recent evidence for the presence of Fc-μ receptors on T-helper cells (41). The immune stimulating effects of IgM in these studies were most clear-cut when suboptimal antigenic stimuli were used, and this may explain why in our strong H-2 incompatible skin graft model this stimulatory activity could not be detected.

In conclusion, we found that IgM does not induce enhancement and that this correlates with its lack of opsonizing activity in the same mouse model. By contrast, enhancing IgG antibodies were strongly opsonizing. This points to an important role for the Fc-fragment in this form of immunosuppression. These findings support the hypothesis of ARCO as an important mechanism of immunologic enhancement.

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CHAPTER 4

ANTIBODY RESPONSE TO ALLOGENEIC AND XENOGENEIC SKIN GRAFTS IN NUDE MICE

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Antibody Response to Allogeneic and Xenogeneic Skin Grafts in Nude Mice

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Abstract

Grafting of allogeneic or xenogeneic skin on nude mice results in a primary antibody response, and during this response the antibody activity switches from IgM toward IgG. We studied the nature of this antibody response in different nude strains (C3H *nu/nu*, C57Bl/6 *nu/nu*, Bl0.LP *nu/nu*, and BALB/c *nu/nu*). At day 21 after transplantation, when antibody titers were falling, the transplantation of a second graft of similar donor type resulted in a secondary antibody response only if the first graft had been removed before the appearance of IgG antibodies. Removal of the first graft after the appearance of IgG antibodies resulted in a nonresponsiveness of the recipient to the second graft. This suggested a suppressive role of the IgG antibodies. This was supported by the finding that passive transfer of nude sera containing specific antibody activity of IgG class suppressed the primary response to a skin graft in the nude mice, whereas sera containing only IgM activity did not induce suppression. We conclude that, in nude mice, an allograft or xenograft induces a primary antibody response that switches from IgM toward IgG, and that the concomitant presence of antigen and IgG antibodies results in the induction of specific unresponsiveness.

Introduction

Congenitally athymic nude mice accept allografts and xenografts permanently (12,13). The absence of graft rejection results from the lack of cytotoxic T cells and not from the absence of a humoral response, because nude mice generate cytotoxic antibodies against allogeneic and xenogeneic skin grafts (4,7,13). Although the antibody is formed in concentrations sufficient to cause rejection

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after administration of rabbit complement (4,7), such rejection does not occur spontaneously in the nude mice because of the inefficiency of the mouse's own complement system in this type of rejection (6).

Earlier we found that in nude mice the antibody activity against allogeneic and xenogeneic skin grafts switched from IgM toward IgG during the primary response (4). In this study we have analyzed this antibody response further, giving special attention to the genetic background of the recipients and the characteristics of the secondary response.

Materials and Methods

Animals. Inbred B10.D2/new Sn mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine) and inbred PVG/c rats from the Institute of Psychiatry, Bethlem Royal Hospital (Beckingham, Kent, U.K.). In our laboratory these strains were kept by continuous brother–sister matings. B10.LP *nu/nu* were obtained from the Radiobiological Institute T.N.O. (Rijswijk, The Netherlands) and C3H *nu/nu*, C57BL/6 *nu/nu*, and BALB/c *nu/nu* were obtained from Gl. Bomholdgard Ltd. (Ry, Denmark).

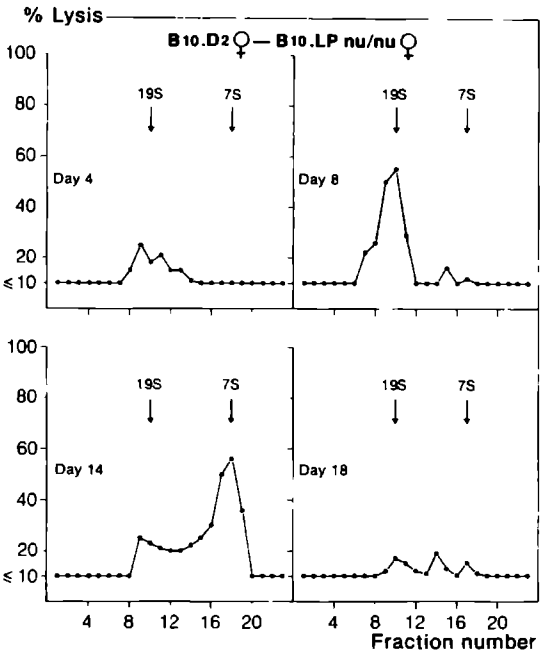

Figure 25-1. Cytotoxic activity of B10.LP *nu/nu* serum after ultracentrifugation on sucrose gradients. Sera were taken at days 4, 8, 14, and 18 after grafting of B10.D2 skin.

Skin grafts. Female tail skin was grafted onto the right dorsal flank of female *nu/nu* recipients by a modified fitted graft technique (2). Donor and recipient mice were between 6 and 8 weeks old. Second grafts were placed onto the left dorsal flank.

Serology. On different days after transplantation nude mice were bled and their sera stored at −90°C. The lymphocytotoxic activity was measured in a trypan blue exclusion test using rabbit serum as a complement source as previously described (3). IgM and IgG antibodies were determined after separation of the sera on isokinetic sucrose gradients (4).

Results

Primary Antibody Response

In different strains of nude mice the primary antibody response was measured by a lymphocytotoxicity assay at various days after grafting of allogeneic B10.D2 or xenogeneic PVG/c skin. As recipients of the xenografts, B10.LP *nu/nu*, C57BL/6 *nu/nu*, C3H *nu/nu*, and BALB/c *nu/nu* were used, whereas in the allogeneic models B10.D2 skin was grafted onto B10.LP *nu/nu* or C57BL/6 *nu/nu* recipients. Analysis of the sera on sucrose gradients showed cytotoxic activity in the 19S as well as in the 7S peak. The 19S activity reached a maximum at day 8 after grafting, whereas the 7S activity was maximal around day 14.

The response of a B10.LP *nu/nu* recipient of a B10.D2 skin graft given in Figure 25-1 is similar to the responses obtained with other donor–recipient combinations of which the results are summarized in Table 25-1.

Secondary Antibody Response

The secondary antibody response to xenogeneic PVG/c skin was studied in B10.LP *nu/nu*, C57BL/6 *nu/nu*, C3H *nu/nu*, and BALB/c *nu/nu* recipients by transplantation of a second PVG/c graft on day 21 after transplantation. The antibody activity was measured on days 4, 8, and 14 after regrafting. Similarly, the secondary response to allogeneic B10.D2 skin was studied in B10.LP *nu/nu*

Table 25-1. Antibody response in nude mice

Donor	Recipient	Maximal titer cytotoxic	Cytotoxic activity at:[a] Day 4 19s	Day 4 7s	Day 8 19s	Day 8 7s	Day 14 19s	Day 14 7s	Day 21 19s	Day 21 7s
PVG/c	B10.LP *nu/nu*	1/256	+	±	++	++	+	++	+	++[b]
PVG/c	BALB/c *nu/nu*	1/64	+	−	++	+	+	++	+	+
PVG/c	C57BL/6 *nu/nu*	1/512	+	−	++	+	+	++	±	+
PVG/c	C3H *nu/nu*	1/64	+	−	++	+	+	++	−	+
B10.D2	B10.LP *nu/nu*	1/256	+	−	+	±	+	+	±	±
B10.D2	C57BL/6 *nu/nu*	1/128	Not done							

[a] Activity in 19s or 7s fraction after separation on sucrose gradients.
[b] Percentage lysis: < 10%, −; 10–25%, ±; 25–90%, +; >90%, ++.

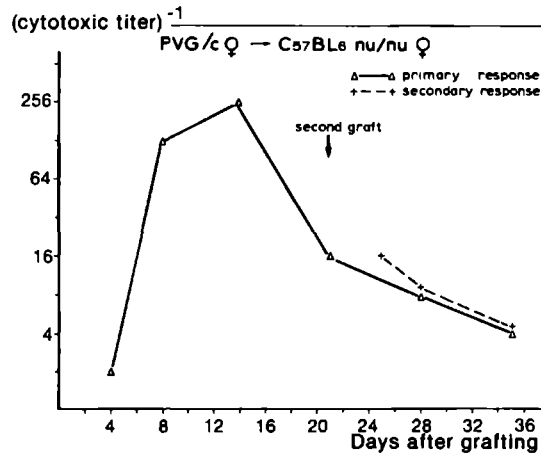

Figure 25-2. Primary and secondary antibody response of C57BL/6 *nu/nu* mice after transplantation of a PVG/c skin and regrafting of a second skin at day 21.

and C57BL/6 *nu/nu* recipients. In none of the cases did antibody activity exceed the residual activity still present after the first transplantation. Regrafting with a third-party graft resulted in a normal response. An example of the absence of the secondary response is given in Figure 25-2.

The absence of a secondary response might be caused by either a generalized immunologic incompetence of nude mice or the induction of suppression by the presence of antigen from the first graft during the production of immunosuppressive antibodies. We therefore studied how graft removal during the primary response influenced the secondary response. A B10.D2 skin graft was removed on days 8, 16, or 21 after grafting. Twenty days after removal of the graft the C57BL/6 *nu/nu* recipients were regrafted with B10.D2 skin. Eight days after regrafting, the animals were bled and the antibody activity was measured. The data given in Table 25-2 show that removal of the graft at day 8, i.e., before the appearance of IgG antibodies, resulted in a strong secondary response. Graft removal on days 16 or 21, i.e., after IgG antibodies had appeared, lead to suppression of this response.

Table 25-2. Abrogation of secondary response by the presence of antigen (B10.D2 → C57BL/6 *nu/nu*)

Removal of first graft on:	Regrafting on	Secondary response at day 8 after regrafting	
Day 8	Day 28	1/512[a]	(21)[b]
Day 16	Day 36	0	(3)
		1/64	(3)
Day 21	Day 41	0	(8)

[a] Cytotoxic titer.
[b] Number of animals tested.

Induction of Suppression by Immune Serum of Nude Mice

We have determined the suppressive capacity of immune sera of nude mice to find out whether the absence of the secondary response was the result of the induction of unresponsiveness by antibodies. C57BL/6 *nu/nu* mice were grafted with B10.D2 or PVG/c skin. On days 8, 16, and 28 the mice were bled and 0.25 ml of this serum was administered intraperitoneally (IP) to C57BL/6 *nu/nu* recipients of B10.D2 or PVG/c skin graft immediately after grafting. The primary response was measured on day 8 after grafting. The results obtained after administration of normal C57BL/6 *nu/nu* serum or immune nude serum are given in Table 25-3. These results show that suppression of the primary response occurred with nude serum obtained 16 days after grafting. This suppression is most likely caused by IgG antibodies and not by IgM antibodies because antiserum obtained at day 8 after grafting, which had a similar or even higher cytotoxic activity but lacked IgG activity, did not alter the alloantibody response. In the xenogeneic combination low amounts of IgG activity were detectable at day 8, and only a slight suppression was observed with this serum.

Discussion

Allogeneic and xenogeneic skin grafts induce a primary antibody response in congenitally athymic nude mice. This is in agreement with the findings of Rygaard (12,13), who found an antibody response to xenografts. The absence of alloantibodies in his study is not in contrast with our findings because he

Table 25-3. Suppression of antibody response by immune nude serum

Treatment[a]	Response at day 8, cytotoxic titer[b]	
A. PVG/c → C57BL/6 *nu/nu*		
None	1/512	(5)[b]
Normal C57BL/6 *nu/nu* serum	1/512	(4)
C57BL/6 *nu/nu* anti-PVG/c serum:[c]		
Day 8 (1/512)[d]	1/128	(3)
Day 16 (1/256)	1/16	(4)
Day 28 (1/16)	1/512	(4)
B. B10.D2 → C57BL/6 *nu/nu*		
None	1/64	(6)[b]
Normal C57BL/6 *nu/nu* serum	1/64	(6)
C57BL/6 *nu/nu* anti-B10.D2 serum:[c]		
Day 8 (1/64)[d]	1/64	(4)
Day 16 (1/64)	0	(3)
	1/8	(3)
Day 28 (0)	1/64	(4)

[a] Intraperitoneal administration of 0.25 ml serum on day of transplantation.
[b] Number of animals tested in parenthesis.
[c] Serum obtained at day 8, 16, or 28 after grafting.
[d] Cytotoxic titer of the serum.

only searched for antibodies at day 30 after grafting. We found that the allo-antibody response has been extinguished by that time.

During the primary antibody response the activity switched from IgM toward IgG. To exclude that this response was dependent on the genetic background of a particular nude strain, nude mice with different haplotypes were used as recipients and in all cases IgM and IgG antibodies were formed.

That the formation of antibodies does not result in graft rejection by nude mice is because of the inefficiency of the mouse complement system, as we have shown earlier (4,7). The finding that nude mice can evoke a primary antibody response to allografts and xenografts leads to the conclusion that major histocompatibility (MHC) antigens are T-cell independent or, alternatively, that MHC antigens need T-cell help, which is provided by a residual T-cell function present in nude mice. This T-cell help should then be located in the very low number of theta-positive cells which are present in nude mice (9,10). Although residual T-cell function cannot be completely excluded it seems more likely that MHC antigens can induce a T-cell-independent antibody response. In keeping with this hypothesis are the findings that the switch from IgM toward IgG in the response to T-cell-independent antigens does not depend on a T-cell function (1,8,11,14).

The findings by Klein et al., (5), who found H-2 antigens being T-cell dependent, are in contradiction with our results. However, in their study they used the absence of 2-mercaptoethanol (2-ME)-resistant antibodies as a criterion for the absence of IgG antibodies. This assumption is probably not completely valid since we have found that IgG is inactivated by 2-ME, especially at low Ig concentrations (3).

In all donor–recipient combinations a secondary response was absent, although the mice could respond to an unrelated second graft. The inability to reject the graft and the occurrence of a primary antibody response results in the simultaneous presence of antigen and antibody, a situation that in normal mice can induce antibody-mediated suppression of the immune response. Two conditions must be fulfilled before one can decide that the absence of a secondary response is the result of an active suppression mechanism and is not a general property of nude mice: (a) The antibodies produced by nude mice must be able to induce suppression after passive transfer. (b) The removal of antigen before the appearance of suppressive antibodies must result in the occurrence of a secondary response. Treatment of nude recipients with immune serum obtained from nude mice after grafting indeed induced suppression of the primary antibody response. It is likely that this suppression is only induced by IgG antibodies and not by IgM antibodies because serum obtained at day 8 after grafting, which had no detectable IgG activity when directed against an allograft or very low IgG activity when directed against a xenograft, induced no or only slight suppression, respectively. This was found in spite of the fact that these sera had the same or even higher cytotoxic titers than the suppressive IgG-containing serum obtained at day 16 after grafting.

The occurrence of a secondary response after removal of the graft before the appearance of IgG antibodies shows that nude mice are able to mount a secondary response and that the absence of this response is a result of antibody-mediated suppression.

We conclude that allografts and xenografts evoke a primary response in nude

mice. This response, including the switch toward IgG, is most likely T-cell independent, although we cannot exclude a possible residual T-cell function being responsible. When concomitantly present with antigen, IgG antibodies induce specific unresponsiveness by which the response is abrogated. In contrast to IgG, IgM antibodies are unable to induce this suppression.

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CHAPTER 5

ENHANCEMENT OF MOUSE SKIN ALLOGRAFTS BY IgG1 AND IgG2 ALLOANTIBODIES AND THE RELATION WITH THEIR OPSONIZING CAPACITY IN VIVO

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SUMMARY

B6AF1 anti-B10.D2 ascites fluid was separated in highly purified IgG1 and IgG2 alloantibodies, which were concentrated to yield similar specific antibody activities on the basis of two-stage lymphocytotoxicity. Their similar specific activities were confirmed by flowcytometric analysis. When tested for enhancement of B10.D2 skin, grafted onto B6JAF1 recipients, and for the opsonization of 51 Cr-labelled B10.D2 leukocytes, IgG1 antibodies were as effective as the IgG2 preparation. Therefore, IgG1 and IgG2 antibodies have similar enhancing capacities.The close correlation with their opsonizing effect, supports the hypothesis that opsonization of antigen-reactive cells (ARCO) is involved in the induction of enhancement.

Administration of alloantibodies directed to the MHC-antigens of a donor graft may, on the one hand, induce prolonged graft survival (Immunological Enhancement), on the other, it may result in antibody-mediated graft rejection (AAR). Using skin allografts in the mouse, we have demonstrated previously that, due to the inefficiency of the mouse complement system, AAR only occurs after the administration of an effective exogenous complement source (1), and that acute rejection is mediated by complement fixing (sub)classes of mouse Ig (2,3), with specificity for class I alloantigens (4). Therefore, strategies to arrive at non-destructive enhancing antibody preparations should be aimed at the isolation of non-complement fixing antibodies and/or the use of antibodies specific for class II MHC-antigens (4).

Enhancement by non-complement fixing IgG1 antibodies was demonstrated in previous studies from our laboratory (2), although this subclass appeared to be 30-50 times less effective than IgG2. However, this comparison was based on the amounts of protein injected, and not on the specific antibody activity present in both preparations. Therefore, we have now compared the enhancing capacity of IgG1 and IgG2 alloantibodies on the basis of specific antibody activity. In addition, enhancing and opsonizing capacities of IgG1 and IGG2 were compared because opsonization of antigen-reactive cells by immune complexes consisting of released graft antigens and administered alloantibody, has been suggested as a mechanism for enhancement (5). This hypothesis implies that enhancing antibodies should have opsonizing capacity.

It will be demonstrated that on the basis of specific antibody activity, IgG1 alloantibodies were as effective as IgG2 antibodies in both enhancement and opsonization.

MATERIALS AND METHODS

Breeding pairs of B10.D2/Sn-new (H-2^d), A/HeJ (H-2^a), and C57BL/6J (H-2^b) were originally obtained from the Jackson Laboratory, Bar Harbor, Me. C57BL/Rij (H-2^b) originated from the Radiobiological Institute TNO, Rijswijk, The Netherlands. (C57BL/Rij x A/HeJ)F_1=B6AF1 and (C57BL/6J x A/HeJ)F_1 =B6JAF1 were raised in the laboratory. BALB.K (H-2^k) mice were obtained from Olac Ltd.Blackthorn, U.K.

B6AF1 anti-B10.D2 ascites fluid was obtained by weekly i.p. injections of a suspension of 5 x 10^7 B10.D2 lymphocytes in complete Freund's Adjuvant, as described earlier (6). Pooled ascites was heat-inactivated at 56°C for 30 min, precipitated with (NH4)2SO4 and after solubilization, sterilized by passage through membrane filters (Schleicher and Schüll, Dassel, West Germany) with decreasing pore sizes ranging from 8µ to 0.2µ.

From this preparation, IgG1 and IgG2 antibodies were isolated by Sepharose-Protein A chromatography (Pharmacia, Uppsala, Sweden) as described by Ey et al (7), and further purified by affinity chromatography on Sepharose-4B, coupled with (sub)class specific antisera (Meloy Labs, Springfield, VA, and Litton Bionetics, Kensington, MD) directed against residual contaminating immunoglobulins. Purified IgG1 and IgG2 were sterilized by passage through a 0.2µ membrane filter. Ig concentrations were determined by radial immunodiffusion with myeloma proteins (Litton Bionetics) as a reference standard.

Specific antibody activity was determined by two-stage lymphocytotoxicity and immunofluorescence. In cytotoxicity experiments, 25 µl B10.D2 splenic leukocytes (5 x 10^6/ml) were incubated with a dilution series of antibody, washed, incubated with anti-IgG, washed again, and incubated with rabbit complement (30 min, 37°C), in a final dilution of 1:12. Direct cytotoxicity of IgG2 was measured in the same assay, but omitting the anti-IgG2 reagent. Fluorescence was determined with an Ortho 50-H flowcytometer (Ortho Instr. Westwood, Mass.), using B10.D2 splenic leukocytes, and T-cells, purified as described by Julius et al (8). Cells (5 x 10^6/ml) were incubated (1 hr, room temperature) with dilution series of antibody and, after washing, with FITC-labelled anti-IgG in PBS supplemented with BSA (1%) and NaN3 (0.1%). Labelled cells were fixed in 1% formaldehyde and analyzed within 3 days. Results

are expressed as Relative Fluorescence Intensities (RFI), which correspond to the fluorescence channels with peak numbers of cells, as determined from the histograms. As second antibodies, goat anti-IgG1 or anti-IgG2 (Meloy) were used for cytotoxicity, and FITC-conjugated goat anti-IgG1 (Meloy) or a mixture of FITC-labelled goat anti-IgG2a and anti-IgG2b (Nordic) for fluorescence.

Opsonization was studied using a liver diversion assay, as described previously (3). 51 Cr-labelled B10.D2 leukocytes, incubated with antibody *in vitro*, were injected i.v. into B6JAF1 mice, and after 3 hr, livers and spleens were removed for gamma counting. Results are expressed as Localization Indices (L.I.), which were calculated by dividing the mean liver/spleen ratio of antibody-treated cells by the mean liver/spleen ratio of untreated cells.

Transplantation of B10.D2 and control BALB.K tail skin onto B6JAF1 recipients was carried out by a modification of the "fitted graft" technique, as described earlier (9). Enhancement was induced by i.p. injections of 0.25 ml antibody on days 0, 2, and 4 after grafting. Dilution series (1:5) were made in 10% normal B6JAF1 serum.

RESULTS

IgG1 and IgG2 were isolated by Sepharose-Protein A chromatography using a continuous, fully automated procedure. The column was charged at pH 8.2, eluted at pH 6.0 for IgG1, and thereafter at pH 2.6 for IgG2 (7). The latter fraction was neutralized immediately using a pH-stat. A total of 250 ml salt-precipitated ascites was run in 5 cycles, and the pooled pH 8.2 effluents, concentrated to their original volume, were rechromatographed once, again in 5 cycles. Residual IgG and other Ig contaminations were removed by affinity chromatography on Sepharose-4B columns, coupled with (sub)class specific antisera against impurities. In this way, highly purified IgG1 and IgG2 fractions were obtained with overall recoveries of 35% and 28% respectively. The preparations were concentrated to yield two-

stage cytotoxicity similar to that of the original ascites (1:320).Their final compositions are shown in Table I.

Table 1. *Concentrations of immunoglobulins (mg/ml)*[a] *in the preparations*

Immunoglobulin	anti-B10.D2 ascites	IgG1 fraction	IgG2 fraction
IgG1	2.32	4.11	0.021
IgG2a	1.85	< 0.001[b]	4.59
IgG2b	1.57	< 0.001	2.53
IgG3	1.78	< 0.001	0.056
IgA	2.82	< 0.002	0.019
IgM	0.67	< 0.002	< 0.002

a) Determined by radial immunodiffusion

b) < : less than the detection limit in double diffusion

After their concentration, the IgG1 and IgG2 preparations gave specific and identical cytolysis curves in two-stage cytotoxicity (Fig.1).

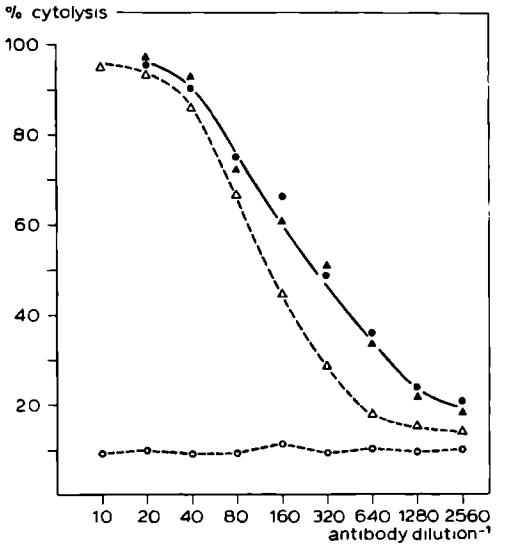

Figure 1. Direct cytotoxicity of IgG1 (0--0) and IgG2 (Δ--Δ) and two-stage cytotoxicity of IgG1 (●—●) and IgG2 (▲—▲) antibodies for B10.D2 target cells.Anti-IgG1 and anti-IgG2 were used in a 1:100 dilution.

This similarity could be demonstrated in a number of experiments in which target cell concentrations or the amounts of anti-IgG antibodies varied. When tested for conventional cytotoxicity only IgG2 specifically lysed B10.D2 cells, with a titer of 1:160, whereas IgG1 was negative (Fig.1).

The similar specific activities of IgG1 and IgG2 were also demonstrated by immunofluorescence. Incubation of B10.D2 spleen cells with diluted (1:80) IgG1 or IgG2 resulted in identical fluorescence histograms, showing a high-intensity subpopulation comprising 60% of the cells, and a population of lower intensity comprising 40% of the cells (Fig.2A). This result suggested that the former population represented the majority of B-cells, and the latter, T-cells. Due to the very low expression of Ia-

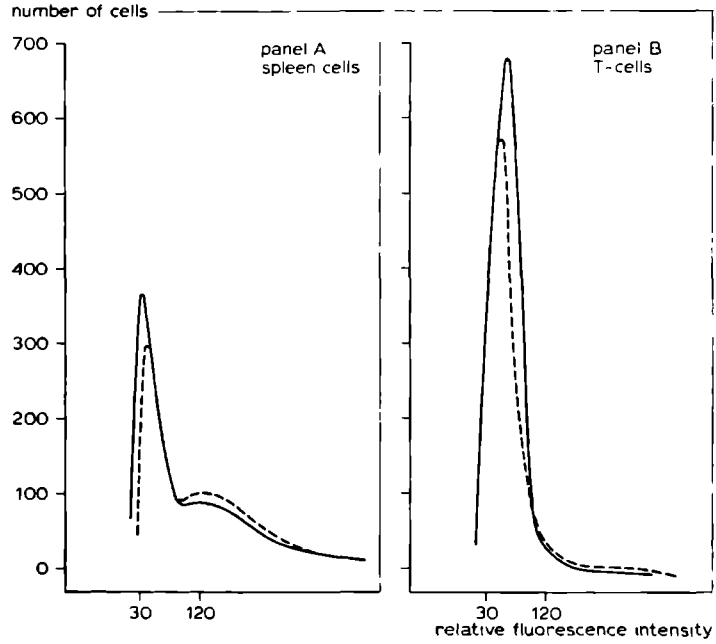

Figure 2. Flowcytometric analysis of B10.D2 spleen cells (Panel A) and B10.D2 T-cells (Panel B), incubated with diluted (1:80) anti-B10.D2 IgG1 (——), or IgG2 (---) alloantibodies. FITC-labelled anti-IgG1 was used in a 1:100 dilution, and FITC-labelled anti-IgG2 consisted of 1:1 mixture of anti-IgG2a (1:25) and anti-IgG2b (1:25). Each histogram represents the analysis of 30.000 cells.

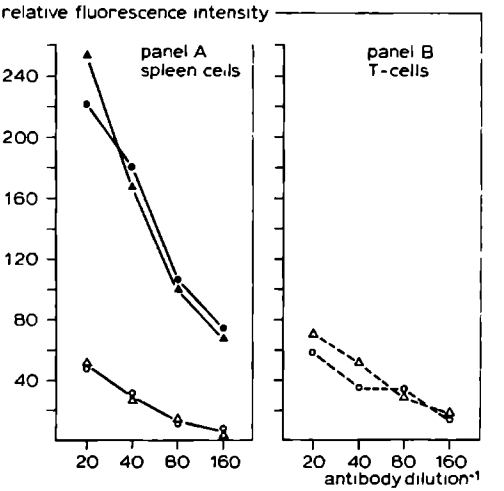

Figure 3. Dose-dependent fluorescence of a high-intensity subpopulation (closed symbols) and a low-intensity subpopulation (open symbols) of B10.D2 spleen cells, incubated with anti-B10.D2 IgG1 (●;○) or IgG2 (▲;△) alloantibodies (Panel A), and of B10.D2 T-cells incubated with IgG1 (○--○) of IgG2 (△--△) alloantibodies (Panel B). Concentrations of FITC-labelled anti-IgG reagents were similar as in figure 2.

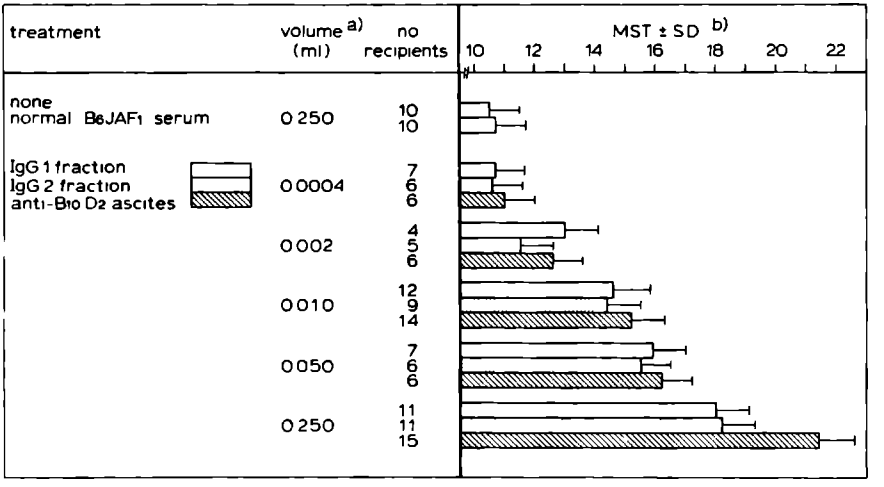

Figure 4. Passive enhancement by B6AF1 anti-B10.D2 ascites and the IgG1 and IgG2 fractions thereof, in B6JAF1 recipients of B10.D2 skin grafts.
a) Injected on days 0, 2, and 4 after grafting, diluted in normal B6JAF1 serum
b) Median Survival Time ± Standard Deviation

antigens, T-cells might only be stained by anti-class I antibodies and therefore, less brightly than B-cells, which can be stained by anti-class I, and by anti-class II antibodies (10,11). To test this possibility, purified T-cells were analyzed, and as is shown in Fig.2B, a single population was visualized, with similar fluorescence as the spleen cell subpopulation of low intensity. Further evidence for this interpretation was obtained by using anti-H-2^k monoclonal anti-class I and anti-class II antibodies. B10.A T-cells demonstrated a single discrete peak of low intensity following incubation with anti-class I,or with anti-class I + anti-class II antibodies. When B10.A spleen cells were reacted with anti-class I antibodies, a discrete peak of similar intensity, though more diffuse, was visualized, but when anti-class I + anti-class II antibodies were used, a second, more brightly staining population was observed (data not shown). These results strongly suggest that the anti-class II activities of IgG1 and IgG2 may be inferred by comparing the fluorescence intensity of the two subpopulations. By plotting the fluorescence channels with peak cell numbers, against the antibody dilutions of IgG1 and IgG2, staining of the two subpopulations in the spleen cells and staining of the T-cells was similar over a broad dose range (Fig.3). This indicated that not only their anti-class I activities were similar, but also their anti-class II activities. The dose-dependent effects of IgG1 and IgG2 were specific. Fluorescence of B6JAF1 control cells incubated with IgG1 or IgG2 did not exceed channel 15, and the data in Fig.3 were corrected for these effects.

Having demonstrated the similar *in vitro* activities of IgG1 and IgG2, their activity to induce enhancement was investigated, and compared with the B6AF1 anti-B10.D2 ascites. As is shown in Fig. 4, graded amounts of all three preparations induced dose-dependent enhancement. Moreover, IgG1 and IgG2 behaved essentially the same, and similar to the ascites fluid from which they originated. Enhancement was specific. When the highest dose of 3 x 0.25 ml was analyzed in the BALB.K → B6JAF1 control combination the normal survival of 12.6 days was not affected by either

IgG1, IgG2 or the ascites pool (data not shown).

Because it has been proposed that opsonization of antigen-reactive cells is involved in enhancement (5), opsonic activity was studied by the liver diversion of 51 Cr-labelled B10.D2 leukocytes, coated with antibody *in vitro*, and injected i.v. into recipient mice. Using suspensions of viable cells without prior incubation with antibody, the percentage of cells moving to the spleen exceeded the number of cells sequestered in the liver, as was demonstrated previously (3). Cells incubated with IgG, however, are diverted away from the spleen, with a simultaneous increased localization in the liver. As is shown in Fig.5, opsonization by IgG1 and IgG2 was specific, and almost identical. With the anti-B10.D2 ascites, similar opsonization was obtained using concentrations which were 5 times lower.

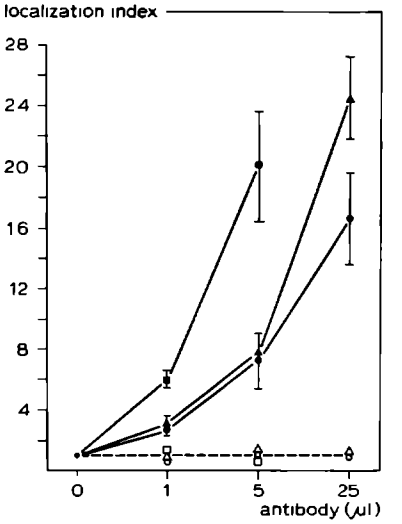

Figure 5. Localization indices in B6JAF1 recipients of B10.D2 leukocytes, incubated with anti-B10.D2 ascites (■-■), IgG1 (●-●), and IgG2 (▲-▲) antibodies, and of control B6JAF1 leukocytes, incubated with the same preparations (open symbols). Each point represents the means ± SD of at least 3 recipients, in 2 separate experiments.

Our results demonstrate that there is a close correlation between the enhancing and opsonizing capacities of purified alloantibodies, and, that IgG1 and IgG2 were equally effective. The effects of IgG1 and IgG2 cannot be due to contaminations. First of all, enhancement and opsonization were specific. Neither the (NH4)2SO4 precipitated ascites, nor the purified IgG1 and IgG2 fractions influenced the survival of control BALB.K skin grafted onto B6JAF1 recipients, and only B10.D2 target cells were diverted to the liver, whereas control cells were not. Secondly, the contaminations of others (sub)classes in purified IgG1 or IgG2 were too small to have significant effects. Contaminating IgG1 or IgG2 were at the highest amounts used, still far below the threshold concentrations that could induce enhancement or opsonization. If the effects were caused by other immunoglobulin contaminants, one would expect enhancement and opsonization to correlate with their concentrations rather than with the amounts of IgG1 and IgG2. This was clearly not the case, and therefore, we conclude that enhancement and opsonization were due to specific IgG1 and IgG2 antibodies.

In our former study on enhancement by IgG1 and IgG2 we found that IgG1 was 30-50 times less effective than IgG2 (2). However, this comparison was based on the amounts of protein injected and if the same is done for the results described here, IgG1 appears to be nearly 2 times more efficient than IgG2. In the analysis of an additional anti-B10.D2 ascites pool, only IgG2 antibodies could be purified, and no specific IgG1 antibodies appeared to be present (data not shown). Thus, depending on the immunization procedure, and the interval between immunization and the collection of serum or ascites, different pools may contain different amounts of specific IgG1 and IgG2 antibody. This notion extends to the specificity of these antibodies. We have demonstrated previously that enhancement across H-2K plus I-region MHC-differences can be mediated by anti-class I (12,13), and by anti-class II antibodies (4). However, the prolongation

of graft survival by anti-class I antibodies was much less than that by anti-class II antibodies. With the latter, enhancement was as efficient as with anti-class I + anti-class II sera (4). Thus, for the comparison of IgG1 and IgG2, analysis of specific antibody activity is indispensable.

Using two-stage cytotoxicity, specific and identical lysis of more than 95% of B10.D2 cells was demonstrated and this indicates equal activities of anti-class I antibody in IgG1 and IgG2. The same conclusion may be drawn from the immunofluorescence analysis of T-cells. The observation that in a spleen cell preparation two subpopulations were visualized, one with similar intensity as isolated T-cells, and a second, more brightly staining population, comprising 60% of the cells, strongly suggests that the latter fraction consisted of B-cells. The more intense fluorescence of this fraction might be due to an increased expression of class I antigens, or to the staining by anti-class I + anti-class II antibodies. The first explanation is unlikely because with monoclonal anti-class I antibodies, no differences were observed between the staining intensities of purified T-cells and spleen cells. Moreover, only after incubation of spleen cells with anti-class I + anti-class II monoclonal antibodies, two subpopulations were observed, similar to the histograms of B10.D2 spleen cells incubated with IgG1 or IgG2 (data not shown). Therefore, binding of anti-class I + anti-class II antibodies is the most likely interpretation. The identical reactivities of IgG1 and IgG2 indicate that both their anti-class I activities and their anti-class II activities were similar.

As was reviewed recently by Voisin (14), controversial results have been obtained about the role of antibody (sub)classes in enhancement. However, decisions against the enhancing capacity of antibodies cannot be made if their specific activity is unknown (15,16). The nature of the graft is also important, because with tumors, cytotoxic effects by IgG2 alloantibodies have been shown to interfere with enhancement (17-19). Our results demonstrate that meaningful comparisons of the effects of IgG1

and IgG2 can only be made with *in vitro* characterized preparations. In this way we could show that IgG1 and IgG2 were equally effective.

The observed opsonizing capacities of IgG1 and IgG2 are in agreement with recent reports from others. Heusser et al (20) have demonstrated that macrophages and macrophage-like cell lines bind aggregated myeloma proteins of IgG1, IgG2a and IgG2b subclass equally well. Similarly, it has been reported that phagocytosis by peritoneal exsudate cells and mouse spleen cells, was nearly as effective with IgG1 as with IgG2, using hybridoma antibodies to sheep red blood cells (21). Also in the human, mouse IgG1 monoclonal anti-T cell antibody was shown to be highly opsonic (22).

The close correlation between the enhancing and the opsonizing capacity of IgG1 and IgG2 supports the concept that opsonization of antigen-reactive cells by immune complexes may be involved in the induction of enhancement (5). We have demonstrated previously that the comparison of IgG and IgM leads to a similar conclusion, because strongly opsonizing IgG antibodies were enhancing, whereas non-opsonizing IgM antibodies were not (3). However, this correlation may not be absolute, and a certain degree of opsonization does not necessarily predict the level of enhancement. Recently, we have compared the enhancing and opsonizing capacities of monoclonal alloantibodies, and demonstrated that monoclonal antibodies were as effective as a conventional alloantiserum in opsonization, but dit not enhance skin graft survival (in preparation). Therefore, opsonic activity may be a prerequisite for enhancing antibodies, but it cannot guarantee that enhancement will invariably occur.

ACKNOWLEDGEMENTS

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CHAPTER 6

IMMUNE REGULATION BY MONOCLONAL ANTIBODIES: FAILURE TO ENHANCE MOUSE SKIN ALLOGRAFTS

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SUMMARY

Monoclonal anti H-2^k alloantibodies were analyzed for their capacity to enhance the survival of B10.A skin, grafted onto B10.D2 recipients. Included were 5 anti-class I and 4 anti-class II antibodies. In contrast to conventional B10.D2 anti-B10.A serum, none of the individual anti-class I or anti-class II monoclonal antibodies induced enhancement. The same negative results were obtained with various mixtures of anti-class I, anti-class II, or anti-class I + anti-class II antibodies. The failure to induce enhancement was not due to inefficient antigen binding *in vivo*, because monoclonal antibodies were as effective as conventional B10.D2 anti-B10.A serum in the induction of acute antibody-mediated graft rejection, and in the opsonization of 51Cr-labelled B10.A leukocytes, injected into B10.D2 recipients pretreated with antibody. These results demonstrate that monoclonal antibodies cannot always substitute for conventional sera, at least not in immune regulation. They also show that, although opsonization may be a prerequisite for the induction of enhancement, it does not guarantee that enhancement will invariably occur.

The administration of antibodies directed against the MHC-antigens of an allograft may have destructive, as well as protective effects (1). These opposite effects, acute antibody-mediated graft rejection (AAR) on the one hand, and prolongation of graft survival (Immunological Enhancement)on the other,have been studied extensively, in order to gain insight into the mechanisms underlying these phenomena, and to define enhancing antibody preparations without destructive activity. To that end, complex allosera have been fractionated in various immunoglobulin (sub)classes, and also according to their anti-class I and anti-class II specificity. However, from recent reviews it is evident that the results obtained in different laboratories are controversial (2,3). This may, at least in part, be due to the high demands for purity which have to be met. The use of monoclonal antibodies would obviate these limitations, because the required specificity and antibody (sub)class can be selected for.

In addition, an analysis of allograft enhancement with monoclonal antibodies might lead to a better understanding of the mechanism of this phenomenon. Previous studies from our laboratory (4,5) and from others (6) have demonstrated that enhancement of allografts is Fc-dependent. Furthermore, since enhancement is specific, it is evident that binding to alloantigens by alloantibody is required. Therefore, complex formation of antigen and alloantibody, and the subsequent interaction of the Fc-part of complex-bound alloantibody with secondary effector systems, appears to be fundamental in the mechanism of enhancement. Several models are compatible with this view.

It has been suggested that antibody might reduce the antigenic stimulus, by the opsonization of donor passenger leukocytes (7). Alternatively, enhancement may involve an effect on antigen-reactive cells (ARC) of the host. Antigen-specific lymphocytes may bind immune complexes consisting of released graft antigen

and alloantibody. Upon binding, the cell may be inactivated, either by the interaction of the Fc-part of the antibody with Fc-receptors on this lymphocyte (2), or antigen-reactive cells may be opsonized by complexes, resulting in their phagocytosis (8). This latter phenomenon has been called antigen-reactive cell opsonization (ARCO).

Two predictions can be made from these models. Firstly, enhancing antibody should be cytophilic and, in case of ARCO or passenger cell opsonization, opsonic. Secondly, enhancement should be a non-determinant specific phenomenon: antibody directed against one or a few determinants on the antigen, should suppress the response to the rest of the determinants on that antigen. In case of passenger cell opsonization, binding to any antigenic determinant will opsonize the whole cell, and will reduce the antigenic stimulus also for those lymphocytes, recognizing determinants different from the one antibody was directed to. Similarly, host lymphocytes can only interact specifically with complexes which display free antigenic determinants and, therefore, antibodies binding to only a few of all determinants of the antigen, will allow the interaction of complexes also with those host lymphcoytes which are directed against different epitopes. Therefore, manipulation of the number of antibody specificities, which can be done using various monoclonal antibodies, may provide further insight into the mechanism of enhancement.

Based on these considerations, we have analyzed the capacity of monoclonal alloantibodies to enhance mouse skin allografts, in relation with their opsonizing capacity. Because the enhancing capacity of conventional IgG2 antibodies is not disputed, monoclonal antibodies of this subclass were selected. It will be demonstrated that monoclonal antibodies were as effective as a conventional alloantiserum in the induction of acute antibody-mediated rejection (AAR) and in opsonization, but that individual anti-class I, anti-class II, as well as various mixtures thereof, did not induce enhancement.

Animals. Inbred strains of B10.D2/Sn-new ($H\text{-}2^d$), B10.A ($H\text{-}2^a$) and BALB/c ($H\text{-}2^d$) were originally obtained from Jackson Laboratory (Bar Harbor, Maine) and C57BL/6Rij mice from the Radiobiological Institute, Rijswijk, The Netherlands. BALB/cBykh ($H\text{-}2^d$) and BALB/cH-2^{dm2} mice were kindly supplied by Dr. P. Démant. BALB.K ($H\text{-}2^k$) mice were obtained from Olac Ltd. Blackthorn, U.K.

Monoclonal antibodies. A total of 9 monoclonal alloantibodies, comprising 5 anti-class I and 4 anti-class II antibodies, was selected for this study. For convenience, they are numbered from 1 to 9 and characteristics are listed in Table I.

Table I. *Monoclonal IgG2 alloantibodies analyzed for their enhancing capacity*

No.	Code	Specificity recognized	γ2-subclass	Cytotoxic[a] titer
anti-class I ($H\text{-}2^k$)				
1.	3-1-3-1	H-2.5	b	5×10^5
2.	11-4.1	$H\text{-}2K^k$	a	5×10^4
3.	H100-27.R55	H-2.25	a	25×10^4
4.	H100-30.R26	H-2.5	b	10^5
5.	H100-5.R4	H-2.11	a	5×10^4
anti-class II($H\text{-}2^k$)				
6.	2-2-1	Ia^k	a	5×10^5
7.	H116-32	Ia.19	b	10^4
8.	11-5.2	Ia.2	b	10^4
9.	11-3.25	Ia.17	b	2×10^2

a) Cytotoxic titer was determined using spleen cells of the skin graft donor strain B10.A

Numbers 1 and 6 were produced in our laboratory, by immunization of BALB/c mice with BALB.K spleen cells and fusion with Sp2/0 Ag14 according to standard procedured described elsewhere (9). Cells of hybrid 11-4.1, producing

no. 2, were kindly provided by the Salk Institute, San Diego, CA. Antibodies 1, 2, and 6 were produced in ascites fluid using BALB/c mice. Antibodies 3, 4, 5 and 7 were generous gifts of Dr. K. Rajewsky. In addition to the characteristics listed in Table 1, detailed information about these preparations can be found in reference 10. Antibodies 8 and 9 were purchased from Becton Dickinson, Mountain View, CA. Further characteristics of these products, and of antibody no. 2 can be found in reference 11. All preparations were diluted in 10% normal B10.D2 serum and sterilized by passage through a 0.2 µ pore size membrane filter (Schleicher and Schüll, Dassel, West Germany).

Conventional antisera. Alloantisera were prepared by weekly intraperitoneal injections of a suspension of 5 x 10^7 lymphoid cells in complete Freund's adjuvant (CFA). This procedure resulted in ascites production within 5-6 weeks. Ascites pools of B10.D2 anti-B10.A, and BALB/c anti-BALB.K were treated with (NH4)2SO4 at 50% saturation and the precipitated proteins, solubilized and dialyzed against PBS, were sterilized by passage through a series of membranes filters (Schleicher and Schüll, Dassel, West Germany) with decreasing pore sizes ranging from 8 µ to 0.2 µ. Anti-lymphocyte serum (ALS) was raised in goats by a subcutaneous injection of 5 x 10^8 C57BL/6Rij lymphocytes suspended in CFA followed by 2 weekly i.v. injections of 5 x 10^8 lymphocytes in saline. Anti-mouse erythrocyte antibodies were removed from the ALS by adsorption with C57BL/6Rij erythrocytes. Antisera used *in vivo* were heat-inactivated at 56°C for 30 min.

Cytotoxic assay. The *in vitro* cytotoxic activity of monoclonal, and of conventional antibodies was determined by trypan blue exclusion with B10.A spleen cells, freed of erythrocytes by treatment with NH4Cl. Using microtiter plates (Greiner, Nürtingen, West Germany), 25 µl of a cell suspension (5 x 10^6/ml) were incubated with 25 µl of a dilution series of alloantibody in Hanks' balanced salt solution supplemented with 0.1% BSA, and 25 µl diluted (1:4) rabbit serum as the complement (C) source,for 30 min at 37°C. The rabbit sera were preselected, and only those sera that lysed 10% or less of the mouse lymphocytes in the absence of alloantibody were used.

Liver diversion assay. The opsonizing capacity of antibodies was analyzed in two ways. B10.A leukocytes, labelled with 51Cr and incubated with mono-

clonal, or conventional alloantibody *in vitro*, were injected i.v. into recipient B10.D2 mice as described earlier (12). In addition, a system was employed in which opsonization as well as the interaction of alloantibody and target cells *in vivo* was analyzed. For this purpose, B10.D2 mice were injected i.p. with alloantibody, 24 hr before the i.v. injection of ^{51}Cr-labelled B10.A leukocytes. Target cells were allowed to react with alloantibody *in vivo*, and opsonization was analyzed after 3 hr, by the removal of livers and spleens for gamma counting. In control experiments, labelled B10.D2 spleen cells were used. These cells were either incubated with anti-B10.A antibody *in vitro*, or injected directly into recipients pretreated with antibody. Doses of antibody are expressed as cytotoxic units (CTU). One CTU is defined as the amount of alloantibody lysing 50% of 1.25×10^5 B10.A leukocytes in the cytotoxicity assay. Results of the opsonization experiments are expressed as localization indices (L.I.). These were calculated by dividing the mean liver/spleen ratio of antibody-treated cells by the mean liver/spleen ratio of untreated cells.

Enhancement of allografts. B10.D2 recipients grafted with B10.A tail skin, as described earlier (13), were injected i.p. with 0.25 ml diluted monoclonal antibody, mixtures thereof, or with conventional allosera, on days 0, 2, and 4 after grafting. The amounts injected were based on the *in vitro* cytotoxic activity with B10.A spleen cells, and they are expressed as cytotoxic units.

Acute antibody-mediated rejection. Acute rejection of well-established grafts on immunosuppressed recipients was induced by i.v. injection of alloantibody together with 0.25 ml rabbit C at day 7 after grafting (14). The recipients were immunosuppressed by i.p. injections of 0.25 ml ALS on days 0, 2, and 4 after grafting. The rabbit serum used as C source was preselected and had no cytotoxic effect on mouse lymphocytes *in vitro*.

RESULTS

Enhancement by conventional alloserum. Since the monoclonal antibodies selected for this study were directed against the H-2^k haplotype, and were produced by BALB/c (H-2^d) mice, a transplantation model was chosen consisting of B10.D2 (H-2^d) recipients

which were grafted with B10.A (H-2^a) donors. In this way, the H-2 barrier consisted of the H-2K and the I-region (15). Untreated recipients rejected donor skin grafts with a median survival time of 10.5 ± 1.1 days, and the injection of 10% B10.D2 serum which was used as a diluent for all preparations had no significant effect on graft survival (Fig.1).

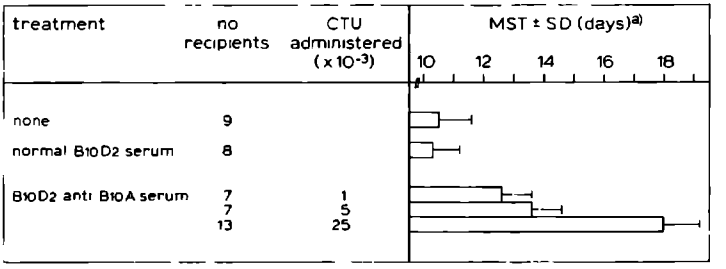

Figure 1. Passive enhancement by B10.D2 anti-B10.A antiserum in B10.D2 recipients of B10.A skin grafts.
a) Median Survival Time ± Standard Deviation. (applies also to Figs.2-4)

Enhancement of B10.A skin, grafted onto B10.D2 recipients has been reported by Staines et al (16), and this is confirmed here. Conventional B10.D2 anti-B10.A serum induced a dose-dependent prolongation of skin graft survival, in comparison with controls (Fig.1). This enhancing effect was specific, because the survival of BALB/c skin grafted onto the H-2L loss-mutant BALB/c H-2^{dm2} was not affected by this antiserum (data not shown).

Effects of monoclonal antibodies on graft survival. The effects of anti-class I monoclonal antibodies on skin graft survival are shown in Fig.2. In only one instance, administration of 1500 x 10^3 CTU of monoclonal no.1, a result suggestive of enhancement was obtained, but a twofold increase in the dose, reduced graft survival to control levels again, as was the case for all other monoclonal anti-class I alloantibodies.
We have previously reported that enhancement by anti-H-2K antibodies across a class I + class II H-2 barrier can be demonstrated, although prolongation of graft survival is small (17,

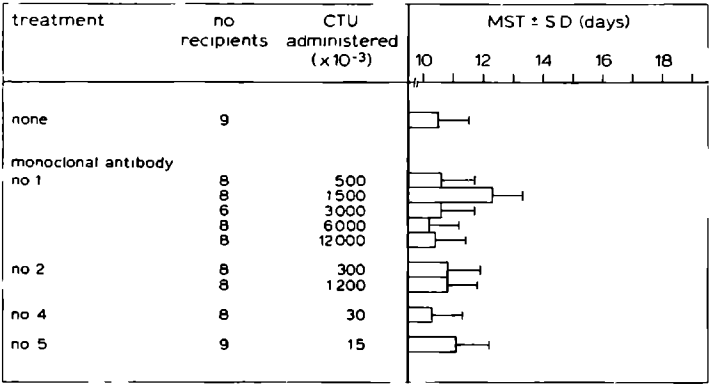

Figure 2. Effects of anti-class I ($H\text{-}2^k$) monoclonal alloantibodies on the survival of B10.A skin, grafted onto B10.D2 recipients

18). On the other hand, enhancement by anti-Ia antibodies across a H-2K + I-region barrier is more readily obtained, as was reported by Jansen et al (19). When we tested the monoclonal anti-class II alloantibodies, graft survival was somewhat prolonged, but none of the antibodies analyzed was able to induce enhancement of a magnitude comparable to that of conventional B10.D2 anti-B10.A serum (Fig.3).

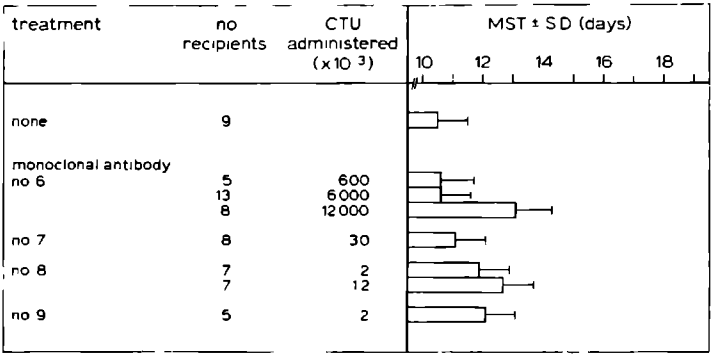

Figure 3. Effects of anti-class II ($H\text{-}2^k$) monoclonal alloantibodies on the survival of B10.A skin, grafted onto B10.D2 recipients

The same held true when various mixtures of anti-class I, anti-class II, and anti-class I + anti-class II antibodies were analyzed (Fig.4).

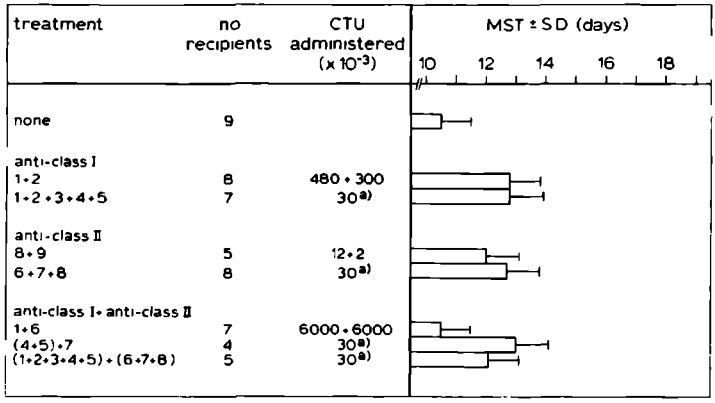

Figure 4. Effects of mixtures of monoclonal alloantibodies on the survival of B10.A skin, grafted onto B10.D2 recipients.
a) Combinations consisting of equal amounts of individual components

Induction of acute rejection (AAR). Although the interaction of monoclonal antibodies with B10.A alloantigens was demonstrated *in vitro*, it is not self-evident that a similar interaction occurs *in vivo*. In order to exclude that inefficient antigen binding *in vivo* was responsible for the lack of enhancing capacity, the interaction of monoclonal antibodies with the graft antigens *in vivo* was tested by their ability to induce AAR. Graded amounts of monoclonal no.2, and of the mixture (1-5 + 6-8), together with 0.25 ml rabbit C were administered i.v. to immunosuppressed B10.D2 recipients of B10.A skin, on day 7 after grafting. The results given in Table II show that preparations of monoclonal antibodies, that had a cytotoxic activity *in vitro* similar to that of the B10.D2 anti-B10.A serum, were equally effective in the induction of complete necrosis of the grafts within 48-72 hr. Because anti-class II antibodies are not capable of the induction of AAR (19), use was also made of 125I-labelled antibodies to demonstrate interaction with the graft antigens *in vivo*. The results of these studies clearly demonstrated the specific binding of both anti-class I, and anti-class II antibodies to the graft antigens (De Waal et al, in preparation).

Table II. *Induction of acute antibody-mediated rejection (AAR) by B10.D2 anti-B10.A antiserum and by monoclonal alloantibodies*

Treatment [a]	CTU administered ($x10^{-3}$)	AAR [b]
B10.D2 anti-B10.A serum	0.16	2/5
	0.8	8/9
	4	5/5
Monoclonal antibody no.2	0.32	2/3
	3.2	3/3
	32	4/4
Monoclonal mixture	0.16	1/5
(1+2+3+4+5) + (6+7+8)	0.8	7/9
	4	5/5

a) AAR is evoked by administration of alloantibody together with 0.25 ml rabbit C to immunosuppressed B10.D2 recipients of B10.A skin, on day 7 after grafting

b) Number of recipients showing complete necrosis of the graft within 48 to 72 hr devided by the total number injected

Opsonization by monoclonal antibodies. Because according to the ARCO hypothesis, opsonization op antigen-reactive cells is involved in the induction of enhancement (8), we analyzed whether the failure of monoclonal antibodies to induce enhancement could be correlated with their opsonizing capacity. For this purpose, the monoclonal mixture (1-5 + 6-8) was compared with the conventional B10.D2 anti-B10.A serum. Opsonization was analyzed by the *in vivo* liver diversion of 51Cr-labelled B10.A leukocytes, coated with graded amounts of monoclonal, or conventional alloantibody *in vitro*. Liver diversion was also studied using uncoated 51Cr-labelled B10.A leukocytes, which were allowed to react with alloantibody injected into the recipient 24 hr before the injection of cells. Since the results with both

assays were identical, only those of the latter are shown. Over a broad dose range, monoclonal antibodies were as effective as B10.D2 anti-B10.A serum in the opsonization of B10.A target cells (Fig.5). Opsonization of uncoated cells in recipients pretreated with antibody therefore, provided in addition to the induction of AAR, a second proof of the interaction of monoclonal antibodies with B10.A antigens *in vivo*. The effects of monoclonal, and conventional B10.D2 anti-B10.A antibodies were specific, because the distribution of B10.D2 control cells was unaffected in both assays (Fig.5).

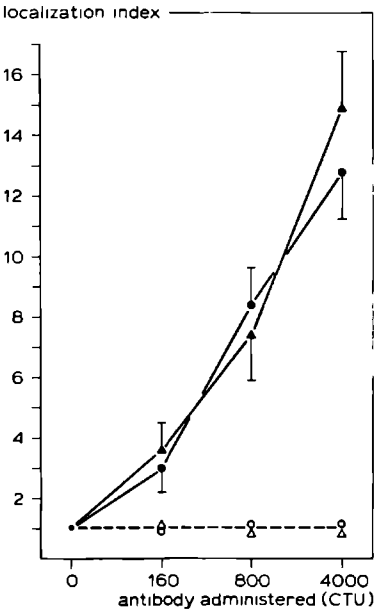

Figure 5. Opsonization of B10.A leukocytes (closed symbols), and of control B10.D2 leukocytes (open symbols), in B10.D2 recipients, injected with conventional B10.D2 anti-B10.A serum (▲-▲) or with a mixture of monoclonal antibodies (●-●). Each point represents the means ± S.D. of 4 recipients, in 2 separate experiments

DISCUSSION

The results of this study demonstrate that although monoclonal antibodies were as effective as a conventional alloserum in op-

sonization and in the induction of AAR, they failed to induce enhancement. In order to explain this failure, their properties have to be considered. The antibodies analyzed are of BALB/c Igh^a allotype and IgG2 subclass. Furthermore, they have, except no.8, a public specificity, and their avidity for B10.A alloantigens is unknown.

It is highly unlikely that the differences in allotypes between the monoclonal antibodies and the conventional alloantiserum, which is of the Igh^b allotype, explains the failure of monoclonal antibodies to induce enhancement, because it has been demonstrated in the rat that even xenogeneic sera are able to enhance kidney allografts (20,21). Moreover, this possibility is ruled out by our own observation that a BALB/c anti-BALB.K serum was as effective as the B10.D2 anti-B10.A serum in the induction of enhanced B10.A skin graft survival in B10.D2 recipients (data not shown).

It is also unlikely that the IgG2 subclass used might explain our results, because enhancement by alloantibodies of this subclass was demonstrated twice in our laboratory in a mouse skin graft model (22,23), and also by others, in the enhancement of mouse tumors (24-30) and rat kidneys (31).

It was also established in our previous studies that enhancement across H-2K + I-region differences cannot only be induced by anti-class II antibodies (19), but also by anti-class I antibodies (17,18), although the effects with anti-class I preparations were relatively weak. These observations were made in models different from the one used in this study, but enhancement with anti-class II antibodies has also been reported using the B10.A→B10.D2 combination (16). Thus, the results of various studies support the observation that both anti-class I and anti-class II antibodies are enhancing (2,3). Because not only the anti-class I, but also the anti-class II and the mixed anti-class I + anti-class II monoclonal antibodies failed to induce enhancement, it is unlikely that the H-2 barrier of the model

has been a responsible factor.

To exclude that the negative results were due to inefficient antigen binding, the activity of monoclonal antibodies was tested *in vitro* and *in vivo*. Antigen binding and activation of rabbit C *in vitro* was demonstrated by lymphocytotoxicity. Interaction with the B10.A antigens *in vivo* was shown by the observation that in terms of cytotoxic units, monoclonal antibodies were as effective as a conventional alloantiserum in the induction of AAR and in opsonization. Moreover, by using 125I-labelled preparations, specific binding of both anti-class I and anti-class II monoclonal antibodies to the graft antigens could be demonstrated (data not shown).

Therefore, the interpretation of our results is first and foremost dependent on unanswered questions concerning the mechanism of immunological enhancement. Two well established phenomena are relevant to the results discussed here. Firstly, the observations that enhancement is Fc-dependent (4-6) and secondly,the notion that antibody directed against one or a few determinants on the antigen, suppresses the response to the rest of the determinants on that antigen (16,32-35). A striking example of the latter phenomenon, having special relevance to our data, is the recent demonstration of rat kidney enhancement by monoclonal antibodies (21,36,37).

It was suggested recently that, in kidney allograft enhancement in the rat, antibody may act by the opsonization of passenger leukocytes, notably the interstitial dendritic cells, thereby reducing the immunogenic stimulus (7). This explanation is compatible with the two phenomena described above. It will undoubtedly also occur in mouse skin allografts since our data showed that 51Cr-labelled B10.A leukocytes were opsonized. Nevertheless, our strongly opsonizing monoclonal antibodies failed to induce enhancement and, therefore, opsonization of passenger leukocytes cannot be a major mechanism in the enhancement of mouse skin, grafted across class I + class II MHC-differences.

This view is in agreement with recent results of Woodward et al (38), who demonstrated that in the case of class I + class II H-2 differences, removal of passenger cells, by using radiation chimeras, did not influence skin graft survival. However, it does not exclude that this mechanism is operative in kidney graft enhancement, and in view of the greater difficulty of enhancing skin relative to a kidney allograft (39), this might explain why kidney allografts were shown to be enhanced by monoclonal antibodies in contrast to mouse skin.

Other hypotheses on the mechanism of enhancement focus on the antigen-reactive cells of the host, which, upon binding to antigen-antibody complexes, may directly be inactivated (2), or may be removed by phagocytosis (8). Our previous data (12,23) showed a strong correlation between the opsonizing and the enhancing capacity of alloantibodies, and therefore, we favour the idea that antigen-reactive cells are opsonized by immune complexes, and are removed by phagocytosis. However,the results reported here indicate that the argument that enhancing antibodies are opsonizing, cannot be turned around. Opsonization may be a prerequisite, but it is no guarantee for enhancement, and other factors, like the nature of the antigen-antibody complexes and the host cells they interact with, will be important as well.

It is not known which antigen-reactive cells, e.g. regulator cells or effector cells, must be eliminated to induce enhancement. If the ARCO hypothesis is right, we must conclude that the complexes of antigen and monoclonal antibodies, presumably formed in our experiments, did not react with the right type of host cells. Some clues may come from recent findings on the composition of conventional anti-Ia antisera. It has been amply documented that Ia-antibodies are most important in the enhancement of organs and tissues grafted across class I + class II differences. These conventional Ia-antisera have now been shown to contain antibodies against unique I-region encoded T-cell products, different from conventional Ia-antigens present on

B-cells (40). Furthermore, evidence has been accumulating for the presence of new, previously unknown class I molecules as well (41). Our panel of monoclonal antibodies did not react with non B-cell Ia-antigens, and it is very well possible that specificities for new class I molecules were lacking as well. Thus, their limited reactivity may explain the failure to induce enhancement.

In conclusion, we have shown that monoclonal antibodies were as effective as a conventional alloantiserum in opsonization and in the induction of AAR, but that they failed to induce enhancement. These results indicate that enhancement is not only dependent on the opsonizing capacity of anti-class I and anti-Ia alloantibodies, but also on their specificity for thusfar unknown H-2 products.

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CHAPTER 7

REJECTION OF LONG-SURVIVING MOUSE SKIN ALLOGRAFTS AFTER WITHDRAWAL OF CYCLOSPORIN A THERAPY

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Rejection of Long-Surviving Mouse Skin Allografts After Withdrawal of Cyclosporin A Therapy

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THE immunosuppressive effects of cyclosporin A (CY-A) as demonstrated in various animal systems[1,2] and in man[3] were recently reviewed by Calne.[4] Most promising thusfar are the results of Green and Allison[5] and of Dunn et al.[6] using rabbit kidney allografts. Recently, Green et al. again demonstrated that extensive prolongation of rabbit kidney allograft survival occurs after a short-term treatment with CY-A.[7] Moreover, the unresponsiveness appeared to be donor-specific and once induced, universal, skin grafts being accepted as well. In mice, treatment with CY-A prolonged skin allograft survival in a model with multiple non-H-2 differences.[1] In a previous report,[8] we have described the influence of CY-A on the survival of mouse skin grafted across a major histocompatibility barrier The immunosuppressive effects of CY-A were demonstrated, but the prolongation of graft survival was clearly dependent on the continuous administration of CY-A. Since this finding differed from the results obtained in the rabbit, we have extended our observations by studying the dose–effect relationships in our model. Furthermore, studies in thymectomized recipients demonstrate that the rejection that invariably occurs after withdrawal of the drug is most likely caused by the recovery of lymphocytes on termination of treatment and not to the appearance of newly matured T cells.

MATERIALS AND METHODS

Inbred lines of B10 D2/new Sn and of A/HeJ mice were originally obtained from the Jackson Laboratory, Bar Harbor, Me C57BL6/Rij mice were obtained from the Radiobiological Institute TNO, Rijswijk, The Netherlands (C57BL6/Rij × A/HeJ)F_1, B6AF_1 hybrids were raised in the laboratory

Tail skin of male B10 D2 donors was grafted onto the flank of male B6AF_1 recipients (H-2^d → H-$2^{a\times b}$) by a modification of the "fitted graft" technique Instead of plaster bandage, a Band-aid was used to cover the graft for 8 days The fate of the grafts was followed by daily macroscopic inspection Grafts were considered to be rejected when no viable epidermis remained The median survival time (MST) and the standard deviations were calculated according to Litchfield,[9] with an arithmatical adaptation Thymectomy was performed in 4-week-old mice according to Herbert[10] Sham-thymectomized animals were prepared by the same procedure except that no thymic glands were removed The animals were used as skin graft recipients 3 weeks after the operation On termination of treatment, successful removal of the thymus was assessed by macroscopic inspection CY-A was dissolved in olive oil at concentrations of 10 and 20 mg/ml by stirring in a 62°C waterbath for 2 hr Once dissolved, the solution could be kept for several weeks at room temperature CY-A doses were based on the mean body weight of the recipients at the onset of the treatment period Usually, the initial body weight averaged 26 5 ± 2 g, increasing to 28–30 g 6 weeks later Oral CY-A treatment was taken very well by all animals, except for a slight weight loss in the first days of treatment

RESULTS

Oral treatment of recipient mice appeared to be most convenient. Daily administration of olive oil as is expressed in group 2 (Table 1) resulted in graft survival times similar to those observed for the untreated controls in group 1. Administration of CY-A was started with a daily dose of 25 mg/kg body weight for 13 days. This protocol resulted in a slight but significant increase in graft survival (group 3). However, using a more extended administration period, all grafts were rejected during treatment (group 4), suggesting that a subop-

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Table 1. Graft Survival With 25 mg/kg Cyclosporin A

Group	Treatment	Graft Survival (Days)	MST ± SD* (Days)
1	None	10 10 10 10 10 11 11 12 12 12	10 3 ± 1.1
2	Olive oil daily from −2	9 10 11 11 11 12 13 13 14	10 8 ± 1.1 (NS)
3	CY-A daily from 0-13	13 13 14 16 16	13 5 ± 1 1†
4	CY-A daily from 0-rejection	14 15 16 18 18	15 2 ± 1 1†

NS, not significantly different from untreated controls

*Median survival time ± standard deviation

†$p < 0.001$ (Student's t test) in comparison with untreated controls

timal dose was given. Therefore, CY-A doses were raised to 75 mg/kg body weight. Significant prolongation of graft survival was demonstrated in the animals receiving CY-A on days −1, 1, and 3 (Table 2, group 5), and administration of CY-A every other day for 17 days resulted in a prolonged survival time of 23.1 days (group 6). With the latter protocol, however, one graft was lost during treatment at day 15, and this again suggested suboptimal conditions. Therefore, daily treatment with 75 mg/kg body weight was adopted, starting 2 days before transplantation. Using this protocol, none of the grafts was rejected during administration, not even during a period of 50 days (group 9). Nevertheless, withdrawal of CY-A resulted in rejection in all experimental groups (groups 7, 8, and 9). Strikingly, the time interval between withdrawal of treatment and rejection in these animals closely equalled the survival times observed in untreated controls.

To exclude that newly matured T cells were responsible for the observed rejection, graft survival was studied in thymectomized and in sham-thymectomized recipients. CY-A was administered at 75 mg/kg body weight for various periods, starting at the day of transplantation. Graft survival times in thymectomized recipients could not be distinguished from graft survival in the sham-thymectomized animals (Table 3), both being similar to the untreated controls receiving only CY-A (Table 2). In each group, some animals received CY-A starting at 2 days before transplantation, but since the results were similar, the survival times with these slightly differing treatment protocols have been put together in the table.

DISCUSSION

The immunosuppressive effects of CY-A in a mouse skin graft model are evident from the data reported here. However, substantial prolongation of graft survival was clearly

Table 2. Graft Survival With 75 mg/kg Cyclosporin A

Group	CY A Treatment (75 mg/kg)	Graft Survival (Days)	MST ± SD* (Days)	Median Interval Between Withdrawal of Treatment and Rejection ± SD (Days)
5	Day −1, 1, 3	13 13 13 13 14 14 15 15 17 19	13 6 ± 1 1	
6	Every other day from −1 to 18	15 22 23 23 24 27 28 31 36	23 1 ± 1.2	
7	Daily from −2 to 8	17 17 17 17 17 18 18 20 20	17 0 ± 1 1	10 2 ± 1.1
8	Daily from 2 to 26	36 36 36 36	35.5 ± 1 0	10 5 ± 1 0
9	Daily from −2 to 51	62 63 64 64 67	63 2 ± 1 0	13 1 ± 1 1

*Median survival time ± standard deviation

Table 3. Graft Survival in Thymectomized and in Sham-Thymectomized Recipients Treated With 75 mg/kg Cyclosporin A

S/T*	CY A Treatment (Daily, 75 mg/kg)	Graft Survival (Days)	MST ± SD† (Days)	Median Interval Between Withdrawal of Treatment and Rejection ± SD (Days)
S	None	10 11 12 12 12 12 13 13 15 15	11 7 ± 1 1	
S	Day 0-13	19 19 20 22 22 24	19 9 ± 1 1	7 9 ± 1 2
	Day 0-26	35 36 37 37 38 39	36 4 ± 1 0	11 4 ± 1 1
	Day 0-51	62 63 63	62 2 ± 1 0	12 2 ± 1 0
	Day 0-101	112 114 122	113 1 ± 1 0	13 0 ± 1 3
T	None	10 11 11 11 11 11 12 12 12 15	11 0 ± 1 1	
T	Day 0-13	18 19 20 21 21 21 23 25	20 2 ± 1 1	8 0 ± 1 2
	Day 0-26	34 35 35 37 38 38 38 38 39 40	36 4 ± 1 0	11 3 ± 1 1
	Day 0-51	62 62 62 62 64 64 64 64 66 67	62 7 ± 1.0	12 7 ± 1 1
	Day 0-101	110		

*S, sham-thymectomized, T, thymectomized

†Median survival time ± standard deviation

dependent on the continuous administration of a sufficient amount of CY-A. Doses of 25 mg/kg administered orally[5,11] or intramuscularly[6] prevented kidney allograft rejection in rabbits and substantially prolonged heart graft survival in pigs. Similar doses injected subcutaneously allowed the establishment of functional histoincompatible marrow grafts in the rat.[12] Our data show, however, that in a mouse skin graft model, graft rejection was delayed but not prevented during treatment with this dose of CY-A. Most likely, this phenomenon again reflects the already well documented differences in the intensity of rejection of various organs and tissues, skin being highly immunogenic in this respect.[13] Nevertheless, if CY-A was administered daily at the higher dose of 75 mg/kg, graft rejection during treatment could be prevented. However, termination of treatment resulted invariably in prompt graft rejection even in thymectomized animals. On the basis of the in vitro toxicity of CY-A for lymphoblasts,[14] it was suggested that CY-A acts in vivo by the suppression of clones of lymphocytes responding to a specific antigenic challenge.[5] The state of specific unresponsiveness observed in rabbits after termination of CY-A treatment might suggest that CY-A not only suppresses, but also eliminates, the responding clones.[7] However, the skin graft rejection we observed in thymectomized and long-term CY-A-treated mice shows that the effect of CY-A is reversible. These differences in responsiveness after termination of CY-A treatment might be explained by the hypothesis that analogous to the different susceptibility of T cells and B cells to CY-A,[15] dose-dependent inhibition could exist within the T-cell lineage as well. Following recognition of antigen, clonal expansion of cytotoxic T cells may be suppressed by CY-A, whereas suppressor T cells are less affected and are able to induce long-lasting unresponsiveness after withdrawal of CY-A treatment. This mechanism may be operative in transplantation models where lower doses of CY-A are effective, i.e., models in which the rejection reaction is less violent than in the one used in our study. In fact, it has been reported that after CY-A treatment in allogeneic bone marrow transplantation in the rat, spleen cells appeared that were suppressive in mixed lymphocyte reaction against donor cells.[12] We conclude that the effectiveness of CY-A treatment depends on the transplantation model used

and that CY-A, at least in mice, does not induce long-lasting transplantation tolerance.

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CHAPTER 8

The first set of experiments described in this thesis deals with the mechanism of immunological enhancement. It was demonstrated that IgG1 and IgG2 alloantibodies were equally effective in enhancement, whereas IgM antibodies were inactive, both in the enhancement of skin allografts and in the suppression of the antibody response of nude mice. In addition, a close correlation could be established between the enhancing and the opsonizing capacities of alloantibodies because enhancing IgG1 and IgG2 antibodies were strongly opsonizing in vivo, whereas non-enhancing IgM antibodies were not. It was further demonstrated that monoclonal alloantibodies were ineffective in enhancement, whereas the effects of antigen-antibody complexes were inferior to the use of antibody alone.

The design of our experiments was based on previous results from our laboratory which demonstrated that F(ab')2 fragments, with similar antigen binding capacities as the enhancing antibodies from which they originated, were unable to induce enhancement (1,2). This finding, which was subsequently confirmed by others (3), showed that enhancement is Fc-dependent. It implied that enhancing antibodies do not act by the simple masking of antigenic determinants, but that the induction of passive enhancement is an active process. Since enhancement is specific, it was also evident that binding of alloantibodies to alloantigen is required. Taken together, these results suggested that the induction of enhancement might result from the complex formation of antigen and alloantibody and, in addition, from the interaction of the Fc-part of complex-bound alloantibody with secondary effector mechanisms. Several models are compatible with this view.

Elimination of passenger cells. It was recently suggested for kidney graft enhancement in the rat, that the prolongation of graft survival resulted from the opsonization of passenger leukocytes, thereby reducing the immunogenic stimulus (4). At first

sight, our results seem to support this hypothesis. We demonstrated the close correlation between the enhancing and opsonizing capacities of various immunoglobulin (sub)classes, and the finding that strongly opsonizing monoclonal antibodies were unable to induce enhancement, could be explained by the assumption that the specificities analyzed reacted with only a limited number of immunogenic graft antigens. Nevertheless, other findings argue against this mechanism. Firstly, the continuous administration of alloantibody should improve the enhancement which is established by the injection of alloantibody at the time of grafting, and this is clearly not the case (5). Secondly, the finding that enhancement by anti-class II antibodies in the case of class I + class II MHC-differences is nearly as effective as with an antiserum directed to the total barrier (6) is in contrast to the hypothesis, because if only the class II antigens had been removed, one would have expected graft survival to be equal to that in combinations with class I differences only. The third and most striking evidence against this mechanism is the recent demonstration of the enhancement of tumors (7), skin, and kidney grafts (8) by anti-TNP antibodies and TNP-coupled alloantigen, because in this situation enhancing anti-TNP antibodies do not interfere with the graft antigens themselves.

Activation of suppressor cells. Other explanations for the phenomenon of enhancement focus on the interaction of antigen-antibody complexes with antigen-reactive cells (ARC) of the host. Antigen-specific lymphocytes may bind immune complexes consisting of released graft antigen and administered alloantibody, and upon binding, the cell may be activated by the interaction of the Fc-part of the antibody with Fc-receptors on this lymphocyte. In this way suppressor cells could be induced. Indeed, the coexistence of enhancing antibodies and suppressor cells was demonstrated in mice, treated for active enhancement (9). Studies from another laboratory, however, failed to demonstrate suppressor cells in rats carrying long term surviving, passively enhanced, kidney allografts (10). Similarly, our results in

nude, T-cell deficient mice argue against a role for T-suppressor cells in the induction of passive enhancement. They also argue against the proposition that suppressive antibody interferes with the cooperation of B-cells with T-helper cells (11).

Inactivation of antigen-reactive cells. Instead of lymphocyte activation by the interaction of the Fc-part of enhancing antibody with Fc-receptors on these lymphocytes, antigen-antibody complexes might inactivate ARC in this way. This mechanism was initially suggested for antibody-mediated suppression of the humoral response (12), and evidence for this model was provided *in vitro*, by showing that in the absence of T-lymphocytes and macrophages, immune complexes could directly suppress the antibody response of B-cells (13). Therefore, antigen-antibody complexes may exert regulatory influences by the interaction of complex-bound antibody with Fc-receptors on antigen-specific lymphocytes.

Fc-receptors have been demonstrated on B-cells and subsequently also on T-cells of various species (14). In man, the presence on T-cells of receptors for either IgG (FcγR) or IgM (FcμR) appeared to be of functional significance, because suppressor cells were shown to carry FcγR, whereas helper T-cells carried FcμR (15). Further studies, however, revealed that FcγR and FcμR were not stable phenotypes and that their expression reflected particular stages of maturation or activation, especially since switches from FcγR to FcμR were observed (16). The functional significance of these markers for the regulation of the immune response, therefore, is still unclear, and the relationship with the phenomenon of antibody-mediated immune suppression remains to be established.

Binding of antigen-antibody complexes to specific antigen-reactive cells, however, may still result in lymphocyte inactivation, but by secondary effector mechanisms which are Fc-dependent (complement, antibody-dependent cellular cytotoxicity

(ADCC), phagocytosis or other). The similar enhancing activities of IgG1 and IgG2 demonstrate that complement cannot play a major role as effector mechanism . The same conclusion was recently reached by others, who showed that decomplementation of graft recipients did not interfere with enhancement (17). Similarly, our observation that IgM was unable to induce enhancement,whereas these antibodies are able to mediate ADCC (18), argues against ADCC as a Fc-dependent effector system. By contrast, the close correlation between the enhancing and opsonizing capacities of alloantibodies strongly suggests, that antigen-reactive cells (ARC) may be eliminated by phagocytosis. Direct evidence for this phenomenon was provided by Hutchinson, who showed that radiolabelled ARC, injected i.v. into recipients treated for antibody-mediated immune suppression, were diverted away from the spleen with a simultaneous increased localization in the liver. This liver diversion was observed with ARC specific for sheep red blood cells, and also in the case of tumor and kidney graft enhancement (19). Further support for this mechanism was provided by others, who showed that the homing of cytotoxic T-lymphocytes to skin allografts in the mouse, was specifically abrogated under enhancing conditions (20).

Thus, the sequence of events taking place in the induction of enhancement may be visualized as follows. Circulating lymphocytes recognize free antigenic determinants in immune complexes which consist of administered alloantibody and released graft antigens. By this process, they may ignore the graft itself and proceed to lymphoid organs where they are eliminated by phagocytic cells, or via the circulation, by the Kupffer cells of the liver. It may be expected that this process goes on as long as these complexes are present. Consequently, pretreatment with antigen-antibody complexes may eliminate a certain number of cells, but due to recruitment, normal numbers of lymphocytes may again circulate at the time of transplantation, resulting in normal graft rejection.

With the implication of antigen-antibody complexes in the in-

duction of enhancement,it is evident that the nature of complex-bound antigen plays a crucial role. Antigens may consist of passenger cells, but also of molecular H-2 products which are shed by passenger leukocytes and by epidermal cells of the graft. Clearly, the free antigenic determinants whithin a complex provide for the interaction with antigen-reactive cells, and the number of different molecular antigens displayed within the complex, will determine the number of ARC-specificities that might be eliminated. All antigenic specificities of the graft barrier are expressed by passenger cells, and antibodies binding to only a few determinants of either class I or class II antigens on these leukocytes, may eliminate all ARC-specificities that respond to the graft barrier. By contrast, a complexed molecular H-2 antigen will eliminate only those ARC which are directed to that particular antigen, and when these types of small complexes would prevail, antibodies against all antigenic specificities of the graft barrier should be administered in order to interfere effectively with the induction of the immune response. Our results with monoclonal antibodies strongly suggest that this is the case. Since a limited number of specificities failed to induce enhancement, it is most likely that some molecular H-2 products were missed, and that a normal response developed against these "uncovered" antigens.

The same explanation applies when it is assumed that different H-2 antigens have different stimulatory capacities, and that the type of antigen-reactive cell that is eliminated is of prime importance. In general, the immunogenicity of individual H-2 loci, whether class I or class II, is the same as judged by skin graft rejection and MLR-reactivity of appropriate recombinant or H-2 mutant mouse strains (21-23). When class I + class II differences exist, however, these effects cumulate to yield a stronger response. This stronger response may occur as a result of summation, or because of an amplification mechanism. Evidence for I-region induced amplification of the cytotoxic response to class I antigens has been provided in vitro (24). Similarly, the superior enhancing capacity of anti-class II as

compared to anti-class I sera suggests that I-region encoded antigens are the major stimulus for graft rejection when class I + class II MHC-differences are present (6). In the light of newly discovered H-2 products, it is very well possible that the panel of monoclonal antibodies that we tested, did not react with strongly stimulatory H-2 antigens.

We have described experiments dealing with the enhancement of mouse skin allografts. In this model, attention is focussed on the induction phase of enhancement since prolongation of graft survival is modest in comparison with tumor enhancement in the mouse, or kidney and heart allograft enhancement in the rat. As a consequence, our results should not be viewed as if the elimination of antigen-reactive cells by antigen-antibody complexes is the only mechanism which is operative in enhanced animals. Different mechanisms may operate in different animals, with different grafts and importantly, in different stages of the immune response. Nevertheless, our results strongly suggest that in the induction phase of enhancement, the opsonization of antigen-reactive cells is of major importance.

Our results with Cyclosporin A (CyA) confirmed the powerful immunosuppressive activities of this agent, but they disproved the original claims that it could induce specific unresponsiveness. Similar findings have now been obtained in other experimental models, where the results with CyA were far superior to existing immunosuppressive regimens notably in outbred species like the mongrel dog and the pig (25). Recent studies indicate that CyA exerts its immunosuppressive effect by interfering with the T-cell dependent production of monokines (IL-1) by accessory cells, and by blocking the receptor expression for these monokines on T-cells, resulting in the abrogation of lymphokine (IL-2) production (26,27). In addition, IL-2 receptive T-cells were rendered unresponsive to this lymphokine, and this sequence of events may explain the high degree of T-cell specificity of CyA. This specificity may provide new approaches in the treatment of T-cell malignancies, since a strong and selec-

tive cytotoxic effect of CyA has already been demonstrated *in vitro* on various T-cell lines and also on freshly explanted malignant T-cells (28). For the moment, however, CyA may first of all "change the face of transplantation"(29).

To date, 1400 transplant patients have been treated with CyA, the majority receiving a kidney (800) or a bone marrow (400) graft (30). In addition, large scale controlled studies are conducted at present in Europe and in North America, and the results thusfar, indicate superior graft survival in comparison with conventional therapy. There is, therefore, every reason for a "cautious optimism" (30) regarding the promises of CyA, although various side-effects, especially its nephrotoxicity, have to be taken most seriously. Nevertheless, CyA offers a new perspective for clinical transplantation, and it is hoped that this agent, or derivatives thereof, may obviate or at least diminish the use of steroids in the near future.

Our studies concerning the mechanism of enhancement offer new prospects in clinical transplantation as well, because it was demonstrated that non-complement fixing, and thus non-destructive IgG1 antibodies were as effective as IgG2 antibodies in the induction of enhancement. However, although knowledge about the world of complex regulatory circuits in immunological processes has expanded considerably, it is still characterized by great blanks. Isolated *in vitro* systems may have adequately been described, but the interplay of initiating, augmenting, helper, and suppressor cells *in vivo*, and their interactions with B-cells, and with idiotypic and anti-idiotypic antibodies, remains largely unknown. Only by further experimentation can more insight be provided into the astonishing ways the immune system operates and thus, into the possibilities to manipulate the immune response specifically.

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De studies die in dit proefschrift zijn beschreven hadden tot doel om meer inzicht te verkrijgen in het werkingsmechanisme van twee experimentele immunosuppressieve behandelingen. Het eerste gedeelte handelt over de transplantaat-beschermende invloed van specifieke anti-donor antistoffen (Immunologische Enhancement), en het tweede over Cyclosporine A, een schimmelmetaboliet. De studies werden uitgevoerd in een muizemodel, waarbij donor staarthuid werd getransplanteerd op de flank van ontvanger muizen. Het immunosuppressief effect van de behandelingen werd bepaald aan de hand van de transplantaatoverleving of door het meten van de door de ontvanger gevormde antistoffen.

Voorgaande onderzoekingen en literatuurgegevens van anderen legden de basis voor de hypothese dat immunologische enhancement door passief toegediende antistoffen tot stand komt via eliminatie van antigeen-reactieve lymfocyten. Onder normale omstandigheden zal kontakt met antigeen met zijn specifieke antigeenreaktieve cel leiden tot stimulatie en clonale proliferatie, maar bij gelijktijdige aanwezigheid van voor het antigeen specifieke antistoffen ontstaan er immuuncomplexen die kunnen leiden tot een Fc-afhankelijke uitdoving, waardoor de reaktie specifiek wordt onderdrukt. Om deze hypothese te onderbouwen werd enerzijds het immunosuppressief effect van antigeen-antistof complexen bestudeerd, anderzijds werd gepoogd inzicht te verkrijgen in de Fc-afhankelijke effektor mechanismen die bij de eliminatie van antigeen-reaktieve cellen betrokken zouden kunnen zijn.

Uit de analyse van het immunosuppressief effekt van verschillende antistof (sub)klassen bleek dat IgG1 en IgG2 antistoffen even effektief waren, maar dat de IgM-klasse noch voor de normale cellulaire respons, noch voor de humorale respons in thymus-loze muizen immunosuppressie kon induceren. Tevens bleek dat het vermogen om enhancement te induceren nauw correleerde met de opsonizerende eigenschappen van enhancing antistoffen.

De rol van antigeen-antistof complexen en de samenstelling daarvan werd onderzocht met monoclonale antistoffen,en met *in vitro* en *in vivo* geformeerde immuuncomplexen. Echter, geen van de geteste monoclonale antistoffen, noch combinaties daarvan induceerde enhancement, terwijl ook met immuuncomplexen bestaande uit toegediend donor-antigeen en conventionele alloantistoffen geen enhancement kon worden geinduceerd.

In het licht van het mechanisme van enhancement wijzen deze resultaten erop dat antistof-gemedieerde immunosuppressie niet behoeft te verlopen via de inductie van T-suppressor cellen, maar direkt, en het meest effektief ingrijpt in de aanloop van de immuunrespons. Gezien de specificiteit van enhancement moet worden aangenomen dat antigeen-antistof complexen hierbij een rol spelen, maar de samenstelling ervan, en het type antigeenreaktieve cel dat in de inductie van enhancement het primaire doelwit vormt, is niet duidelijk. Wel kan worden geconcludeerd dat de Fc-afhankelijkheid van enhancement betrekking heeft op het vermogen van de antistof om fagocytose te initiëren, en daarmee ondersteunen onze resultaten de hypothese dat enhancement tot stand komt via de eliminatie van specifiek antigeenreaktieve cellen.

Onze bevindingen met Cyclosporine A wijzen er op dat dit middel een sterke, hoewel niet-specifieke, immunosuppressie kan induceren. Deze gegevens zijn inmiddels bevestigd door andere laboratoria, en de resultaten van recente klinische trials in verschillende centra geven aan dat Cyclosporine A, of hieraan verwante middelen, in de nabije toekomst het gebruik van steroiden overbodig zou kunnen maken. Echter, ook onze studies naar het mechanisme van enhancement geven een nieuw perspektief voor de klinische transplantatie, omdat kon worden aangetoond dat niet-complement bindende, en dus niet-destruktieve IgG1 antistoffen even effektief zijn als IgG2 antistoffen in de inductie van enhancement.

Dit proefschrift is tot stand gekomen dankzij de vruchtbare samenwerking van de medewerkers van de afdeling Nierziekten, met name op het laboratorium, en de inzet van velen daarbuiten. De discussies over de resultaten van het onderzoek, het gezamenlijk congresbezoek en de diepgaande nabesprekingen daarvan, en de betrokkenheid van medewerkers van de afdeling Pathologische Anatomie, vormden een immunologische lego doos waarmee naar hartelust gespeeld werd. Dat de ontworpen luchtkastelen tot ware proporties werden teruggebracht is te danken aan de inzet van allen, maar met name dankzij het solide experimentele werk van Wim Tamboer, Truus Rijke, Cor Jacobs, en Jeanne van Rijs. Hetzelfde geldt voor Hans Beck, Desirée Jacobs en Peter Hendriksen, die in het kader van hun laboratoriumstage bij verschillende aspecten van het onderzoek waren betrokken. Het dierexperimentele werk kwam tot stand dankzij de voortreffelijke inzet van Jacqueline Hagemann en Peter Daamen, in de loop van de jaren bijgestaan door Trees Borggreve en Eric Schroevers, die voor dit onderzoek alleen al meer dan 500 transplantatiegroepen inzetten en talloze antisera produceerden en registreerden. Ine Cornelissen van het laboratorium Chemische Pathologie van de afdeling Pathologische Anatomie was in voorkomende gevallen altijd bereid om op zeer deskundige wijze als "stand-in" te funktioneren. Jan Koedam, Lia van de Vorle-Houben, dhr. G.M. Busser en de overige medewerkers van afdeling IV en van de boerderij van het Centraal Dieren Laboratorium waren hier ten nauwste bij betrokken vanwege hun zorg voor de proefdieren en hun hulp. Hetzelfde geldt voor Gerrie Grutters die het radioactieve werk in de muis in goede banen wist te leiden. Hierbij wil ik tevens Magda van de Manacker en mevr. A.C. Felten-Chardon betrekken die ik erkentelijk ben voor hun hulp bij het verkrijgen van radioactieve chemicaliën. De figuren in dit proefschrift werden met de grootste zorg getekend door medewerkers van de afdeling Medische Illustratie (hoofd: dhr. W.P.J. Maas), en de foto's werden vervaardigd door medewerkers van de afdeling Medische Fotografie (hoofd: dhr. A.T.A. Reynen). De medewerkers

van deze afdelingen ben ik ook erkentelijk voor het vervaardigen van grote aantallen dia's, met name Dhr. A.T. van Uden voor zijn verzorging van het instant-werk en de posters. De heer E. de Graaff (hoofd Medische Bibliotheek) en zijn medewerkers ben ik dankbaar voor hun hulp bij het verzamelen van de literatuur; dit geldt met name Gerry Hermkens van de bibliotheek Medische Microbiologie. Het typen van dit manuscript en de talrijke versies daarvoor werd met grote toewijding en nauwkeurigheid verricht door Ilse Hilgers-Biermans en Erna Kokke; dankzij hun inspanningen werden de krappe tijdslimieten ook nu weer gehaald. Ik vind het bijzonder leuk dat Margriet Lems de omslag wilde verzorgen, en met het resultaat feliciteer ik haar en mezelf. Tenslotte wil ik Barbara, mijn lief, bedanken voor de essentiële inbreng die zij in de loop der jaren in dit werk heeft gehad; haar idee voor de omslag valt daarbij in het niet.

CURRICULUM VITAE

Simon Pieter Meeuwis Lems is op 4 januari 1949 geboren te Rotterdam. Hij bezocht de Christelijke HBS en MMS "Charlois" (tegenwoordig: Christelijke Scholengemeenschap "Maarten Luther") te Rotterdam en behaalde het einddiploma HBS-b in 1966. Daarna studeerde hij scheikunde aan de Vrije Universiteit te Amsterdam. Het kandidaatsexamen in de richting S2 werd behaald in 1970. Voor het doctoraalexamen werd een uitgebreid hoofdvak verricht op de afdeling Biochemie (Prof.dr.R.J. Planta) en een algemeen tentamen Radiochemie (Prof.dr.B. van Zanten). In het kader van het bijvak Immunochemie werkte hij gedurende 6 maanden op de Immunochemische afdeling (Prof.dr.K.W.Pondman) van het Centraal Laboratorium van de Bloedtransfusiedienst (CLB) te Amsterdam. Het doctoraalexamen werd behaald in mei 1973. Vanaf maart 1973 was hij werkzaam als wetenschappelijk medewerker op de afdeling Immunochemie van het CLB, waar hij op een beurs van het Ministerie van Volksgezondheid en Milieu Hygiëne een produktie gericht onderzoek verrichtte naar de ontwikkeling van immunologische bepalingsmethodieken voor marihuana produkten en LSD. Vanaf oktober 1977 is hij werkzaam op het laboratorium van de afdeling Nierziekten van de Universiteitskliniek voor Inwendige Ziekten van het Sint Radboudziekenhuis te Nijmegen. Tot november 1979 werkte hij middels subsidie van de Universitaire Onderzoeks Pool (projektnummer G1-77) aan de bereiding van transplantaatbeschermende (enhancing) antilichaamfrakties met behulp van affiniteitschromatografie aan transplantatie antigenen. Tot juli 1982 werkte hij, gesubsidieerd door de Stichting voor Medisch Wetenschappelijk Onderzoek FUNGO (projektnummer 13-29-43), aan het mechanisme van transplantaatbescherming door antistoffen, het onderwerp van de hierbeschreven studies. In juli 1982 verwierf hij een subsidie van de Nier Stichting Nederland om onderzoek te doen naar de regulatie van de immuunrespons tegen huidtransplantaten bij de muis met behulp van monoclonale antistoffen en immuuncomplexen.
Hij is getrouwd met Barbara van der Brugge en zij hebben drie kinderen, Margriet, Floris en Nicolien.

STELLINGEN

I

IgM antistoffen hebben geen transplantaatbeschermende (enhancing) werking en missen tevens het vermogen om te opsonizeren.

II

De transplantaatbeschermende effekten van IgG1 en IgG2 alloantistoffen van de muis zijn vergelijkbaar en nauw gecorreleerd met hun opsonizerende aktiviteit.

III

Het vermogen van alloantistoffen om te opsonizeren is een voorwaarde, maar geen garantie voor hun transplantaatbeschermende aktiviteit.

IV

Complexen van passagère donor leukocyten en transplantaatbeschermende antistoffen spelen geen rol in de induktie van Immunologische Enhancement van huid-allotransplantaten in de muis.

V

Bij het testen van Immunologische Enhancement in pre-klinische modellen van proefdieren met een adequaat complement systeem verdient het gebruik van niet-complement bindende IgG alloantistoffen de voorkeur boven de toepassing van anti-Ia sera, teneinde een interferentie door antistof gemediëerde destruktie uit te sluiten.

Marquet RL, van Es AA, Heystek GA et al: Transplantation *25:* 188, 1978

VI

Gezien de antistof respons die in thymusloze nu/nu muizen wordt opgeroepen moeten tenminste de klasse I H-2 antigenen als thymus onafhankelijk worden gekenmerkt.

VII

Indien bij klinische niertransplantaties Cyclosporine A wordt toegepast moet dit middel gedurende de eerste 10 dagen post-operatief via continue intraveneuze infusie worden toegediend, en wel in zodanige hoeveelheden dat de bloedspiegel op 200 ng/ml wordt gehouden om voldoende immunosuppressie te garanderen met minimale toxische effekten.

White DJG, Calne RY: Transplant Proc *15:* in press

Kahan BD, Ried M, Newburger J: Transplant Proc *15:* in press

VIII

Onder invloed van een cellulaire immuunrespons komen klasse II H-2 antigenen tot expressie op het capillaire vaatendotheel van huid-allotransplantaten bij de muis. Dit suggereert dat het vaatendotheel, en niet de passagère Ia-positieve leukocyt, fungeert als doelwit voor transplantaatafstoting in geval van een klasse II transplantatie barrière.

de Waal RMW, persoonlijke mededeling

IX

Het verdient aanbeveling om na te gaan of conjugaten van antigeen en cytotoxische geneesmiddelen of toxinen antigeen-specifieke immunosuppressie *in vivo* kunnen induceren.

Vitetta ES, Krolick KA, Uhr JW: Immunol Rev *62:* 159, 1982

Volkman DJ, Ahmad A, Fauci AS et al: J Exp Med *156:* 634, 1982

X

De basale mechanismen die ten grondslag liggen aan de induktie en de regulatie van de immuunrespons kunnen pas dan begrepen worden als een gedetailleerde kennis beschikbaar is van de chemische strukturen van de moleculen die de cellulaire interakties van immunocompetente cellen mediëren.

XI

De suggestie van Highton et al dat adsorptie met proteine A-Sepharose van sera van patienten met Rheumatoïde Arthritis informatie geeft over de hoeveelheid circulerende IgG-IgM(Rf) complexen is onjuist, omdat ook niet gecomplexeerde IgM(Rf) kan binden aan Sepharose-proteine A gebonden IgG.

Highton J, Panayi GS, Shepherd P et al: Ann Rheum Dis *40:* 575, 1981

Faaber P, persoonlijke mededeling

XII

Het feit dat de frequentie van peritonitis bij chronische cyclische peritoneaal dialyse (CCPD) lager is dan bij chronische ambulante peritoneaal dialyse rechtvaardigt de toepassing van CCPD-behandeling ondanks de hogere kosten.

Diaz-Buxo JA, Farmer CD, Walker PJ et al: Artif Organs *5:* 157, 1981

XIII

De benaming "*in vitro*", als tegenhanger van het begrip "*in vivo*", dient vervangen te worden door de term "*in plastico*".

XIV

Het is te betreuren dat de groep Boney M de aanzet tot vernieuwing van het kerkelijk lied na de eerste poging met Psalm 137 niet krachtiger heeft doorgezet.

Boney M: The rivers of Babylon

XV

Voor een heilzaam gebruik van de bijbel is het raadplegen van een bijsluiter onontbeerlijk.

Labuschagne CJ: Wat zegt de bijbel in GODS naam ?, Boekencentrum, 1977

Rapport over de aard van het Schriftgezag "God met ons": Kerkinformatie 113, Leusden, 1981

XVI

De benaming "demissionair" voor een kabinet dat slechts lopende zaken behartigt suggereert ten onrechte dat een "missionair" kabinet uitsluitend beleidsplannen met visie ontwikkelt.

XVII

Door de plaats die promovendi in de corona van de aula van de Katholieke Universiteit te Nijmegen innemen, moeten zij zich in allerlei bochten wringen om de oppositie op behoorlijke wijze van repliek te dienen.

Simon P.M. Lems

Nijmegen, 25 november 1982