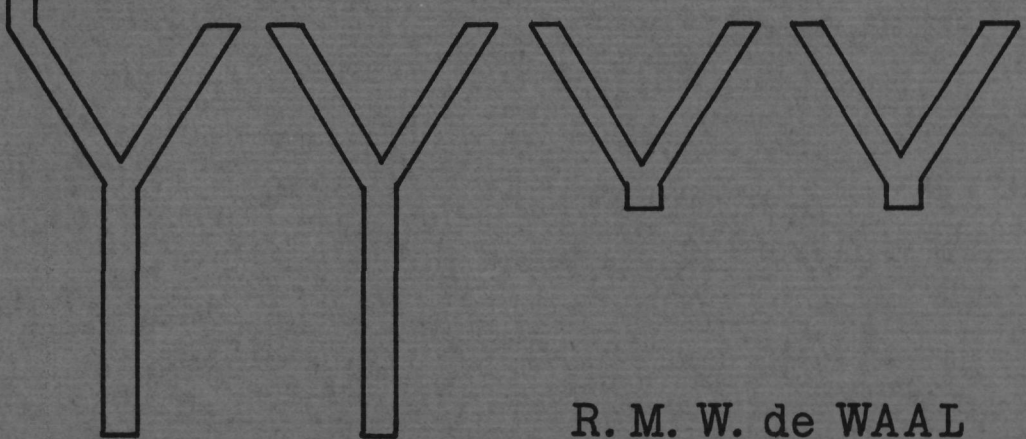
THE ROLE OF PURIFIED ANTIBODIES AND ANTIBODY FRAGMENTS IN THE ENHANCEMENT AND THE ACUTE DESTRUCTION OF MOUSE ALLOGRAFTS



R. M. W. de WAAL

The role of purified antibodies and antibody fragments in the enhancement and the acute destruction of mouse allografts

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PROEFSCHRIFT

ter verkrijging van de graad van doctor in de
Wiskunde en Natuurwetenschappen aan de
Katholieke Universiteit te Nijmegen,
op gezag van de Rector Magnificus
prof.dr. P.G.A.B. Wijdeveld volgens besluit van
het College van Decanen in het openbaar te verdedigen
op donderdag 20 december 1979
des namiddags te 2 uur precies

door

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geboren te Dongen

Druk: Centrale Reprografie Directoraat A-faculteiten
Universiteit Nijmegen

Dit proefschrift werd bewerkt op de afdeling Nierziekten (hoofd: Prof.Dr. P.G.A.B. Wijdeveld) van de Universiteitskliniek voor Inwendige Ziekten (hoofd: Prof.Dr. C.L.H. Majoor) en op het Instituut voor Pathologische Anatomie (hoofd: Prof.Dr. G.P. Vooys) van het St. Radboudziekenhuis te Nijmegen.

Dit onderzoek werd gesteund door de Nier Stichting Nederland, de Stichting voor Medisch Wetenschappelijk Onderzoek FUNGO, en door een subsidie uit de Universitaire Onderzoekspool.
Het proefschrift werd uitgegeven met steun van de Nier Stichting Nederland.

Ieder die tot aan het tot stand komen van dit proefschrift heeft meegewerkt wil ik van harte bedanken.
Met grote bekwaamheid werd het dierexperimentele werk verricht door Jacqueline Hagemann, later op even uitstekende wijze door Ine Cornelissen. Gedeelten van het laboratoriumwerk werden deskundig verricht door Riki Willems, Wies van Gorp en Ans Borgonjen. Ton van Sommeren en Henny Roelofs leverden in het kader van hun laboratoriumstage eveneens een bijdrage.
In hoge mate waardeer ik de mogelijkheid die mij werd gegeven, het onderzoek als medewerker van de afdeling Pathologische Anatomie (hoofd: Prof. Dr. G.P. Vooys) af te ronden. Alle medewerkers van dit Instituut die hieraan door hun steun of belangstelling hebben bijgedragen, in het bijzonder de medewerkers van het laboratorium Chemische Pathologie, wil ik gaarne dank zeggen.
De medewerkers van afdeling IV (hoofd: dhr. J. Koedam) van het Centraal Dierenlaboratorium (Hoofd: Dr. W.J.I. van der Gulden) assisteerden op deskundige wijze bij het dierexperimentele werk.
De stimulerende discussies met de medewerkers van het laboratorium Nefrologie waren zeer leerzaam; de steun en begeleiding uit deze groep waren onontbeerlijk.
De figuren in dit proefschrift werden gedeeltelijk vervaardigd door medewerkers van de afdeling Medische Illustratie (hoofd: dhr. W. Maas). De overige figuren werden getekend door Marjo Beijnvoort. De medewerkers van de afdeling Medische Fotografie (hoofd: dhr. A. Reynen) vervaardigden de foto's. Bij het verzamelen van literatuur waren medewerkers van de Medische Bibliotheek (hoofd: dhr. E. de Graaf) behulpzaam. De heer Jaq. Akkers (afd. Reprografie Directoraat A-faculteiten) gaf deskundig advies bij het vermenigvuldigen van dit proefschrift. Het typen van het manuscript werd met grote toewijding verricht door Janny van Rennes.
Mijn ouders wil ik van harte bedanken voor de steun die zij mij door de jaren heen hebben gegeven. Dit proefschrift mag ook als de kroon op hun werk worden gezien. Tenslotte wil ik Paula, mijn vrouw, bedanken voor haar inbreng in dit werk, welke weliswaar moeilijk te omschrijven, maar toch essentiëel is geweest.

Aan Paula, Sabine en Tim,
mijn ouders.

GLOSSARY

Allograft : transplantation between two individuals of the same species.

Autograft : transplantation in which donor and recipient are the same individual.

ALS : anti-lymphocyte serum.

Enhancement : specific antibody-mediated suppression of the cellular immune response to allografts; *passive-*: by administration of antibodies to the recipient; *active-*: by antibodies formed by the recipient.

Haplotype : set of genetic determinants located on a single chromosome.

Histocompatibility : compatibility as determined by transplantation.

Opsonization : process of coating an antigen which facilitates the phagocytosis of the antigen.

Specificity (antigenic): antigenic determinant defined by the specific reaction with an antiserum.

Xenograft : transplantation across a species barrier.

CONTENTS

GENERAL INTRODUCTION

History

Transplantation as a method to replace irreversibly damaged tissues or organs has stimulated the imagination of man since a long time. In the years before 1900, several reports were published on successful transplantation of animal or human tissue, mainly dealing with skin grafts. (Baronio, 1804; Bunger, 1823; Bert, 1863).
In those days no distinction was made between the behaviour of auto-, allo-, or xenografts, which by itself can be regarded as an indication that the over-all results were very poor. Technically successful renal transplantation was first described in 1905 in dogs (Carrell and Guthrie, 1905; Floresco, 1905). At best, the kidney grafts survived for 8 days, and this disappointing result was thought to be due to surgical complications.

In subsequent years, it was noticed, that autografts could survive indefinitely, whereas allo- and xenografts ceased to function after several days. In 1944, Medawar described his experiments with repeated transplantations in rabbits. He showed that skin, grafted onto sensitized recipients, was rejected in an accelerated fashion. This "second-set" reaction proved that allograft rejection had an immunological basis (the immunity theory, advanced in 1908 by Bashford et al.) and that it was not a lack of "growth substance" in the transplanted tissue that made it die (the "athrepsia" theory, advanced by Ehrlich in 1906). Medawar also observed that during the rejection episode the allografted tissue was heavily infiltrated by mononuclear cells. Mitchison showed in 1953 that the immunity to skin grafts could be transferred to another animal by injection of "sensitized" lymphoid cells, and not by injection of serum. Cellular mechanisms, therefore, were supposed to be of predominant importance in transplantation immunity (reviewed by Billingham and Silvers, 1963; and Brent and Medawar, 1967). Humoral immunity was thought to play a negligible role.

More recent experiments showed, however, that rejection could also be induced by transfer of serum from sensitized animals to recipients of organ or skin grafts (reviewed by Stetson, 1963; and by Carpenter, d'Apice and Abbas, 1976). Antibody-dependent rejection was readily induced in recipients of primarily vascularized organ grafts. Recipients of skin grafts were much more resistant to this form of rejection. This difference is due to the accessibility of the graft to antibody. It was shown (Gerlag et al., 1975) that skin allografts in the mouse become sensitive to the action of humoral factors such as antibodies only at day 4 or 5 after transplantation.
Another prerequisite for the induction of antibody-mediated acute rejection of allografts is the presence of an active complement system (French, 1972). In species like dog and rabbit, such an active system is present. In the mouse, the endogenous complement route is not activated efficiently enough by alloantibodies directed against the graft to evoke rejection. Acute rejection can only be induced in murine recipients if a heterologous effective complement, like rabbit or guinea pig complement, is administered along with the antiserum (Koene et al., 1973; Berden et al., 1977).

In man, preformed antibodies can destroy an organ allograft in a hyper-acute way, showing that alloantibodies are able to cause activation of the human complement system. This phenomenon was described first by Kissmeyer-Nielsen et al. (1966).
Antibodies that are formed after transplantation can also harm renal allografts in man. Busch et al. (1969) associated chronic rejection with deposition of IgG on the vascular endothelium. This type of rejection is characterized by progressive intimal proliferation and vessel wall thickening with resultant ischemia (Kindcaid-Smith, 1967), and it is only seen if cellular mechanisms are depressed by immunosuppressive drugs. Chronic rejection by itself, however, responds very poorly to immunosuppressive therapy and accounts for a considerable amount of graft losses. It is thus obvious that antibody-mediated mechanisms can play an important role in graft destruction.

Evidence has emerged from the field of tumor immunology that antibodies

can be also active in graft protection. The initial observations were made in rodent tumor systems in the early 1900s (Flexner and Jobling, 1907). When a non-viable fibrosarcoma was inoculated into rats some days before a challenge with viable tumor, the challenge inoculum, instead of regressing, grew progressively and this often led to the death of the recipient animal.
Casey introduced the word "enhancing" for this phenomenon and showed that it had an immunological basis (Casey, 1934). It was found that enhancement was related to histocompatibility antigens (Snell, 1946) and that is was mediated by antibodies (Kaliss and Kandutsch, 1956). Not only tumor grafts, but also grafts of normal tissue and organs (Billingham et al., 1956; Stuart et al., 1968) including skin grafts in mice (Jeekel et al., 1972; McKenzie and Snell, 1973) could be passively enhanced by alloantibodies.

It is thus clear that both destruction and protection can be brought about by alloantibodies. The conditions that determine which of either phenomenon will occur in a given transplantation situation are not entirely clear. We have studied this problem in a mouse skin allograft model in which both antibody-mediated rejection and protection can be reproducibly induced.

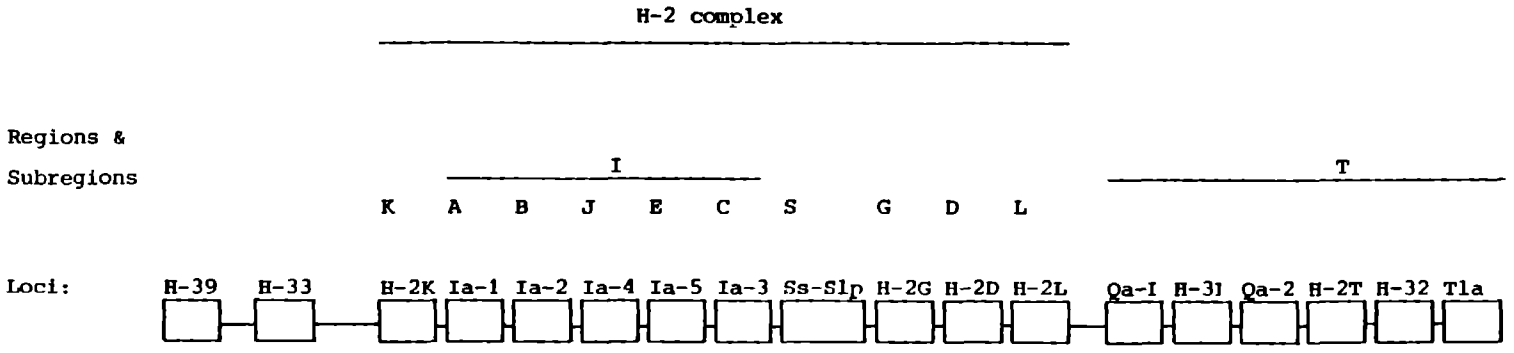

Fig. 1. Genetic map of the H-2 complex and its vicinity. Only one locus for each region or subregion is shown. The loci are as follows: H-histocompatibility. Ia-I region-associated antigen. Ss-serum serological. Slp-sex-limited protein; Qa-Q region antigen; Tla-thymus-leukemia antigen. The order of H-2D, H-2L and of the T-region loci is not known; the arrangement shown is arbitrary (adapted from J. Klein et al., 1978).

Transplantation genetics of the models used

The investigations described in this thesis were performed mainly in a mouse skin allograft model. In some of the experiments a mouse fibrosarcoma was used as the donor tissue. Donor and recipient mice were inbred and genetically well defined. The basic immunogenetics and immunobiology of the mouse major histocompatibility system, the H-2 complex, will not be discussed here, since excellent reviews are available (Klein, 1975; Festenstein and Démant, 1978). This system is very complex as a consequence of the polymorphism of the individual loci. However, to define the strain combinations and antisera used, and to determine the actual antigenic differences that give rise to the cellular and humoral immune responses in the recipients, the following inventarization has to be given.

The genetic map of the H-2 complex, situated on chromosome 17, is shown in fig. 1. In this complex, several transplantation loci have been described. Transplantations across differences in these loci result in fast rejection of the graft in the case of a major locus incompatibility and in delayed rejection in the case of a minor locus incompatibility. The major histocompatibility loci are present in the K- and D-regions, and in the IA-subregion. A minor locus is present in the IC-subregion. Recently, another histocompatibility locus was reported to be present in the vicinity of the H-2D locus (McKenzie et al., 1977), which was called H-2L. The inbred mouse strains used in this study had the genotypes depicted in table I. In the $H\text{-}2^a$ haplotype, a crossover is present between the IE- and IC-subregions. Using this strain as a recipient for B10.D2 or B10.BR skin, the influence of parts of the H-2 complex in histocompatibility can be investigated. In the same combinations antibodies can be raised and the role of these antibodies in graft protection or destruction can be studied. To minimize non-H-2 differences, the B6AF1 hybrid was raised from A/HeJ and C57Bl parents. The H-2 specificities, coded for by the K, D, and L-regions, which can be serologically detected on all cells in the different haplotypes used, are given in table II. B6AF1 mice carry the specificities both from A/HeJ and C57Bl mice.

TABLE I

Genotypes of the mouse strains used

Strain	H-2 haplotype	K	I					S	G	D/L
			A	B	J	E	C			
B10.D2	$H\text{-}2^{d}$	d	d	d	d	d	d	d	d	d
B10.BR	$H\text{-}2^{k}$	k	k	k	k	k	k	k	k	k
A/HeJ	$H\text{-}2^{a}$	k	k	k	k	k	d	d	d	d
C57Bl	$H\text{-}2^{b}$	b	b	b	b	b	b	b	b	b

TABLE II

Serologically detectable H-2 antigenic specificities of the mouse strains used

strain	Haplotype	Specificity																					
B10.D2	H-2^{d}	-	-	3	4	-	6	-	8	-	-	-	13	-	-	-	-	-	-	-	-	-	-
B10.BR	H-2^{k}	1	-	3	-	5	-	7	8	-	11	-	-	-	-	-	-	-	-	-	-	-	-
C57 Bl	H-2^{b}	-	2	-	-	5	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/HeJ	H-2^{a}	1	-	3	4	5	6	-	8	-	11	-	13	-	-	-	-	-	-	-	-	-	-

Strain	Haplotype	Specificity																					
B10.D2	H-2^{d}	-	-	-	-	27	28	29	-	31	-	-	34	35	36	-	-	-	40	41	42		
B10.BR	H-2^{k}	23	24	25	-	-	-	-	-	-	32	-	-	-	-	-	-	-	-	-	-		
C57 Bl	H-2^{b}	-	-	-	-	27	28	29	-	-	-	33	-	35	36	-	-	39	-	-	-		
A/HeJ	H-2^{a}	23	24	25	-	27	28	29	-	-	-	-	-	35	36	-	-	-	40	41	42		

Strain	Haplotype	Specificity																					
B10.D2	H-2^{d}	43	44	-	46	47	49	-	-	-	-	-	-	-	-	-	-						
B10.BR	H-2^{k}	-	-	45	-	47	49	-	-	52	-	-	-	-	60	-	-						
C57 Bl	H-2^{b}	-	-	-	46	-	-	-	-	-	53	54	-	56	-	62	-						
A/HeJ	H-2^{a}	43	44	45	-	47	49	-	-	52	-	-	-	-	60	-	-						

TABLE III

Ia antigens expressed by the mouse strains used

Strain	Haplotype	Specificities																	
B10.D2	H-2^{d}	-	-	-	-	-	6	7	8	-	-	11	-	-	-	15	16	-	-
B10.BR	H-2^{k}	1	2	3	-	-	-	7	-	-	-	-	-	-	-	15	-	17	18
C57 Bl	H-2^{b}	-	-	3	-	-	-	-	8	9	-	-	-	-	-	15	-	-	-
A/HeJ	H-2^{a}	1	2	3	-	-	6	7	-	-	-	-	-	-	-	15	-	17	18

Strain	Haplotype	Specificity																	
B10.D2	H-2^{d}	-	-	-	-	23	-	-	-	-	-	-	W30	-	-	-			
B10.BR	H-2^{k}	19	-	-	22	-	-	W25	W26	-	W28	-	-	31	32	33			
C57 Bl	H-2^{b}	-	20	-	-	-	-	-	-	-	-	W29	W30	-	-	-			
A/HeJ	H-2^{a}	19	-	-	22	-	-	W25	W26	-	W28	-	-	31	32	33			

TABLE IV

Specifities recognized in the strain combinations

Donor	Recipient	H-2 antigens	Ia antigens
B10.D2	B6AF1	31, 34	11, 16, 23
B10.BR	B6AF1	7, 32	-

The I-region associated specificities, the socalled Ia antigens, are listed in table III. Ia antigens are present mainly on B-lymphocytes, some T-lymphocytes, and on macrophages.
The specificities that are recognized in the combinations used are shown in table IV. In B6AF1 anti B10.D2 serum, antibodies against H-2K.31 and 34, and against Ia.11, 16 and 23 are present. The H-2K.34 specificity is probably an Ia specificity (Staines et al. 1976).
The B6AF1 anti B10.BR serum contains antibodies against H-2 specificities 7 and 32. H-2.32 is coded for by the D region of the H-2 complex; H-2.7 is coded for by the G region. Antigens that are coded for by this latter region are expressed on the membranes of erythrocytes and do not play an important role in histocompatibility. Thusfar no antibodies against Ia antigens have been raised in this combination.

Enhancement and acute rejection of mouse skin allografts

The experimental conditions for the reproducible induction of acute antibody-mediated rejection and passive enhancement were established in the B10.D2 → B6AF1 model (Koene et al., 1973). Both antibody-mediated effects could be elicited by administration of the same B6AF1 anti B10.D2 serum. Passive enhancement can be induced by administration of 0.25 ml alloantiserum on days 0, 2, and 4 after transplantation; acute rejection is evoked by administration of 0.25 ml alloantiserum to ALS-immunosuppressed recipients, along with 0.25 ml rabbit serum as a source of complement on day 7 after transplantation.
Attempts to separate the enhancing and destructive activities in the serum by using physicochemical methods failed (Jansen et al. 1975a). Neither was it possible to make a separation by isolating pure IgG subclass proteins (Jansen et al., 1975b). Antibodies that were exclusively enhancing could be isolated when they were purified on the basis of the specificity against which they were directed (Jansen et al., 1975c). Antibodies directed against Ia antigens showed unimpaired enhancing activity in comparison to the unfractionated alloantiserum, but were unable to induce acute rejection. To prepare these anti-Ia antibodies, advantage was taken from the fact that red blood cells do not carry I-region products on their membranes. By absorption with red blood cells from the donor strain, antibodies directed against products of the K-region were removed. Along with this procedure, the destructive activity vanished.

Although these studies gave a better insight into the mechanisms of antibody-mediated graft destruction and protection, several questions remained unanswered, and some new problems were raised. These formed the basis of the current investigation and they are described in detail in the following paragraph.

Aim of this study

The first question that was raised from the results of the previous studies concerned the activity of anti H-2K and anti H-2D antibodies in enhancement and acute rejection. To test the *in vivo* activities of these socalled SD (serologically defined) antibodies, an isolation procedure had to be designed.
A method for the elution of anti H-2K and H-2D antibodies from murine blood platelets was described in brief by Colombani et al. (1976). Staines et al. (1976) described a procedure for the elution of anti SD antibodies from murine erythrocytes. In this latter protocol, the eluted antibodies were purified by ammonium sulphate precipitation and used for *in vitro* serologic testing. We found that the antibodies isolated in this way were not suited for *in vivo* injection due to the considerable release of hemoglobin during the elution procedure. In chapter 1, a method is described for the elution of anti H-2K antibodies from murine red blood cell ghosts. In this way, disturbing hemoglobin release during the elution step is diminished, and the eluate is, after another purification step, suitable for *in vivo* testing.

The *in vivo* testing of the isolated anti SD antibodies is described in chapter 2. Both anti H-2K and H-2D antibodies were tested for their destructive and protective activities on murine skin allografts.

A second question that was raised concerned the practical usability of enhancing antibodies. Large scale production of these antibodies will be necessary, before they can be used in clinical treatment protocols. The preparation of anti Ia sera by absorption with red blood cells is very cumbersome. Large amounts of red blood cells or platelets of defined H-2 phenotype are required. During elution potentially pathogenic immune complexes might be released. To be able to prepare antibody fractions of defined specificity on a larger scale, other techniques have to be developed.

A possible method of purification is affinity chromatography on immobilized

transplantation antigens. If it were possible to isolate and purify H-2K, D antigens, and to couple them covalently to a carrier particle, the antigen could be used as an immunoabsorbent to prepare Ia antisera.

To test the practical usefulness of such a purification procedure we tried to purify B6AF1 anti B10.D2 antiserum on an affinity column of coupled B10.D2 transplantation antigens. We used the detergent NP-40 to solubilize the H-2 antigens from mouse lymphocytes, according to the procedure described by Schwartz and Nathenson (1971).
We will not discuss here the structure of the products of the K, D, and I regions; an extensive review on this subject was recently published by Vitetta and Capra (1978).
In chapter 3 the isolation, purification and coupling of transplantation antigens is described. The *in vitro* and *in vivo* activities of the antibodies eluted from the antigen column are also described in this chapter.

Up till now we concentrated on the preparation of enhancing antiserum fractions by attempts to separate the antibodies on the basis of their distinct antigen-binding specificities. Another, and possibly even simpler, approach might be to change the antibody molecules themselves in such a way that they would loose their destructive capacity with preservation of the enhancing activity. This goal might be accomplished by the use of F(ab')2 fragments of antibodies. These fragments can be prepared from IgG by removal of the Fc part (fig. 2).
The results of Koene et al. (1973), Gerlag (1975) and Berden et al. (1977), suggest that the activation of the classical pathway of the complement system is of major importance in the induction of the acute rejection phenomenon, although there are some indications (Berden, unpublished) that complement-independent mechanisms can play a role. Since activation of the classical complement route occurs via the binding of the first complement component, C1q, to the Fc part of the IgG molecule, we assumed that it would not be possible to evoke acute rejection by administration of F(ab')2 fragments. It was known already that F(ab')2 fragments lack *in vitro* complement-dependent cytotoxicity.

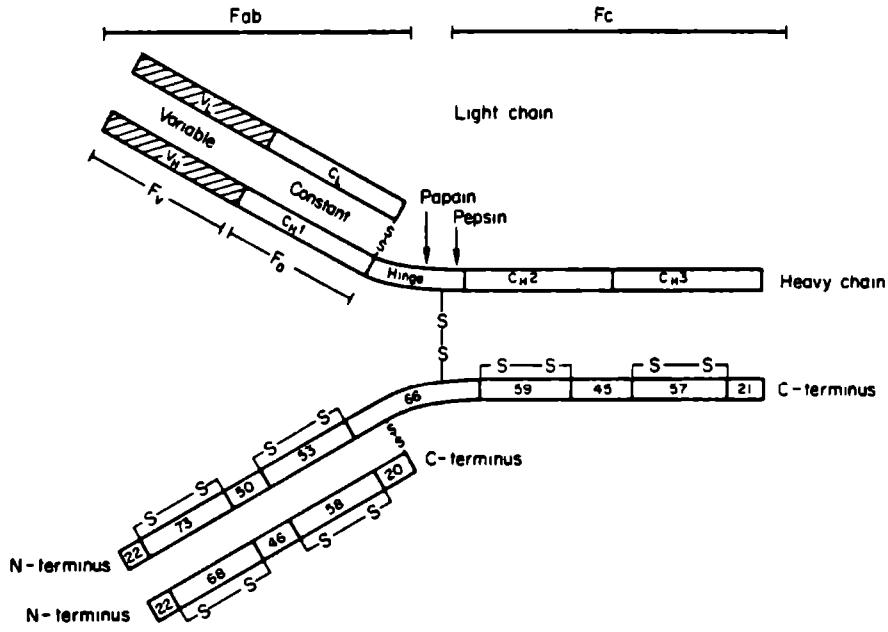

Fig. 2. A typical IgG molecule. The cleavage by proteolytic enzymes occurs in the hinge-region, resulting in the formation of F(ab) or F(ab')2 fragments.
In the lower part of the figure, the number of amino acids in typical domains is shown.

The antigen-binding site of the IgG molecule is unaffected by the removal of the Fc-fragment. It is, therefore, likely that administration of F(ab')2 fragments to recipients of allografts will result in the covering of graft antigens with F(ab')2 fragments.

The diminished accessibility of the graft to the host's cellular defense mechanisms as the result of masking of donor transplantation antigens in this way, was regarded as a possible mechanism of enhancement.
On the basis of these assumptions, F(ab')2 fragments would have enhancing activity comparable to intact IgG, but lack the capacity to evoke acute rejection. We decided therefore, to prepare these fragments and test their *in vitro* and *in vivo* activities. The results obtained in the skin allograft model are described in chapter 4.

A model in which enhancing activity of F(ab')2 fragments actually was reported is tumor allotransplantation in mice (Kaliss et al., 1976). As this finding was not in agreement with the results obtained by us in the skin allograft model, we decided to test the effects of similarly isolated and purified F(ab')2 fragments in a tumor allograft model. This model was developed during this study, and proved to be very suitable for the determination of putative enhancing activity, since graft protection leads in this model to progressive growth of the tumor. The *in vivo* effects of F(ab')2 fragments on tumor allografts are described in chapter 5.

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Chapter 1

PROCEDURE FOR THE ISOLATION OF MOUSE ANTI H-2 ANTIBODIES FROM COMPLEX ALLOANTISERA

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Chapter 1 was published in the Journal of Immunological Methods 1977
Vol. 14, page 225

B6AF1 anti B10.D2 ascites fluid was incubated with donor strain red blood cells to absorb the anti H-2 antibodies. The antibody-coated RBC were then lysed, and from the ghosts the antibodies were eluted. This anti H-2 preparation was further purified by affinity chromatography, using an immobilized anti IgG2 antiserum. Thus a pure anti H-2 IgG2 was obtained for *in vitro* and *in vivo* testing.

Many mouse alloantisera have been shown to contain antibodies directed against at least two different groups of transplantation antigens. These antigens are encoded by genes in the K and D regions and the I region of the major histocompatibility complex (MHC), respectively. They differ in tissue distribution, molecular weight and function. Antibodies against I region associated antigens (Ia antigens) can be isolated from complex alloantisera by absorption with mouse red blood cells, that carry the H-2K and H-2D antigens but lack Ia antigens. By such absorption anti-H-2K and H-2D antibodies are removed from the sera. There is recent evidence, that these purified anti Ia sera can induce specific immunosuppression leading to prolonged or even indefinite survival of allografts in several transplantation models (Davies et al., 1974; Staines et al., 1974; Jansen et al., 1975a). This phenomenon is called immunologic enhancement. It is of great interest to test also the activity of pure preparations of the anti-H-2K and H-2D antibodies, that remain absorbed onto the erythrocytes. *In vitro* and *in vivo* studies show (Jansen et al., 1975a) that these antibodies play a role in graft destruction at the efferent arc of the immune response. However, studies of Staines et al. (1975) suggest that they might have some enhancing activity.
To test this, pure preparations of anti H-2 antibodies, uncontaminated by anti Ia antibodies are required. We present here a method for the elution of a pure anti H-2 IgG2 antibody fraction from mouse erythrocytes, which is suitable for *in vitro* and *in vivo* testing. The IgG2 subclass is chosen because antibodies of this subclass can induce enhancement as well as accelerated graft destruction (Jansen et al., 1975b).

MATERIALS AND METHODS

Alloantiserum

Inbred strains of B10.D2 n/Sn(H-2^d) mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine). C57B16/Rij (H-2^b) and A/HeJ(H-2^a) mice were obtained from the Radiobiological Institute (Rijswijk, The Netherlands). In our animal laboratory, these mouse strains were kept by brother-sister matings. (C57B16 / Rij x A/HeJ)F1 (=B6AF1) hybrids were produced in the laboratory. The alloantiserum was prepared by injecting, intraperitoneally, B6AF1 mice at weekly intervals with 5 X 10^7 B10.D2 lymphoid cells obtained from spleen, thymus and lymph nodes, made into a suspension in complete Freund's adjuvant (equal volumes of suspension and adjuvant). After five injections, the mice developed ascites and could be tapped every 1 to 2 weeks. The cytotoxic titer of the ascites fluid was the same as the titer in the serum of these mice. Antisera were stored at -20°C. This antiserum contains antibodies directed against the K and I regions of the MHC and we have previously shown that after removal of the anti H-2K antibodies the enhancing capacity of this serum remains unimpaired (Jansen et al., 1975a).

Affinity chromatography

Specific antiserum against mouse 7S IgG2 was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the following procedure: 6 ml of specific antiserum were mixed at room temperature with an equal volume of saturated ammonium sulphate (SAS) at pH 6.5. The resulting precipitate was washed three times with 50% SAS dissolved in 6 ml 0.1 M $NaHCO_3$ and 0.5 M NaCl, and dialysed against the same solution overnight. The ratio of CNBr-Sepharose and protein that would give optimal adsorption capacity was determined by coupling protein, precipitated from 6 ml antiserum (dissolved in 6 ml 0.1 M $NaHCO_3$ 0.5 M NaCl) to 0.75, 2.0, and 4.0 g CNBr-Sepharose respectively. Although 4 g CNBr-Sepharose 4B coupled far more protein, the adsorption capacity appeared almost nil.

Best results were obtained with 0.75 g CNBr-Sepharose 4B. This amount was swollen on a glass filter and washed for 15 min with 10^{-3} M HCl. Thereafter, protein (about 100 mg) was coupled to the gel under gentle stirring. Coupling was completed in 2h. Uncoupled protein was mixed twice more with the same quantity of activated Sepharose 4B. After coupling, any remaining active groups in the gel were inactivated by incubating the gel with 1 M ethanolamine at pH 8.0 for 1 h. To remove the non-covalently adsorbed protein after coupling, the gel was washed following a modification of van Munster's method (Van Munster, 1972), successively with (a) 0.1 M borate buffer at pH 8.0 + 1 M NaCl, (b) 0.1 M citrate buffer at pH 6.0., and (c) 0.05 M citrate buffer pH 2.6 + 1 M NaCl. Thereafter, the gel was equilibrated with 0.05 M citrate buffer at pH 6.0. The Sepharose carrying the antibodies was packed in jacketed glass columns (2.6 x 10 cm), cooled with tap water.

Serology

In vitro cytotoxic activity was measured with B10.D2 and C3H/He lymphoid cells using the trypan blue dye exclusion method (Gorer and O'Gorman, 1956). The C3H/He strain was chosen to test the specificity of the alloantiserum. This strain has the haplotype H-2^k and bears no antigenic determinants, against which the alloantiserum is directed. One tenth milliliter of spleen cells, prepared free of red cells with Tris-NH_4Cl (5 x 10^6 cells/ml), was incubated in tubes with equal volumes of antibody dilution and rabbit complement for 30 min at 37°C. Percentages of lysed cells were read microscopically.

RESULTS AND DISCUSSION

Absorption procedure

B10.D2 red blood cells (RBC) were purified from blood by the following procedure: 100 ml of freshly drawn blood, collected in CPD medium, were centrifuged at 700 g for 20 min, and the supernatant and buffy coat were

discarded. The pellet was washed three times in 0.16 M phosphate buffered saline at pH 7.2. Thereafter the RBC were further purified by passage through a closely packed cotton wool column (20 x 4 cm) as described by Diepenhorst et al. (1972). In this way, more than 99% of leukocytes and platelets were removed. Ten milliliters of full antiserum (IgG2 content 4 mg/ml) were absorbed with these purified RBC during 2 h at room temperature in sealed tubes, rotating end over end at 15 rpm.

Recovery procedure

After incubation the RBC were recovered and washed extensively with PBS. To avoid disturbing haemoglobin release during the elution procedure, the RBC were first lysed by mixing with a Tris-NH_4Cl solution. The IgG carrying ghosts were precipitated by centrifugation (22,000 g, 10 min), washed and resuspended in elution buffer, consisting of 0.05 M citrate buffer pH 2.6 and 1 M NaCl. The ghosts were precipitated by centrifugation and the supernatant was recovered and quickly brought to pH 7.4. The eluate was concentrated by ultrafiltration (Amicon ultrafiltration membrane XM-50), and dialysed overnight against 0.05 M citrate buffer pH 6.0 which served as the starting buffer for affinity chromatography. The dialysed eluate was applied to the anti IgG2 column in portions of 3 ml. The column was washed with 0.05 M citrate buffer, pH 6.0, at a flow rate of 30 ml/h, to remove unbound material. Adsorbed protein was eluted with 0.05 M citrate buffer pH 2.6, containing 1 M NaCl. The protein containing eluate was collected immediately in 0.25 Tris-HCl buffer at pH 8.6, to avoid loss of biologic activity. A typical elution pattern is shown in fig. 1. Before use, all fractions were equilibrated in saline by dialysis, concentrated by ultrafiltration, and sterilized by means of membrane filtering (Sartorius Membran Filter SM 11306, Göttingen, West Germany). The IgG2 content of the final preparation was 10 µg/ml.

The yield of the described procedure is about 0.1 mg of protein per 50 ml of packed RBC. Immunodiffusion showed the preparation to contain only antibodies of the IgG2 subclass. Fig. 2 shows the cytotoxic activity of the eluted antibodies as a function of dilution. It can be seen that

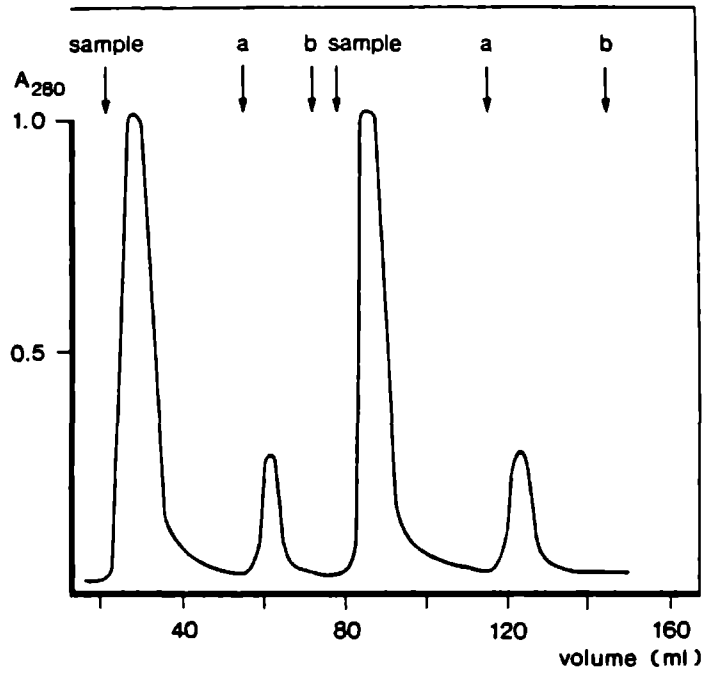

Fig. 1. Part of an elution profile obtained by the separation of mouse IgG2 from mouse anti H-2K IgG, eluted from mouse red blood cell ghosts. At (a) bound protein is eluted. At (b) the column is re-equilibrated with starting buffer.

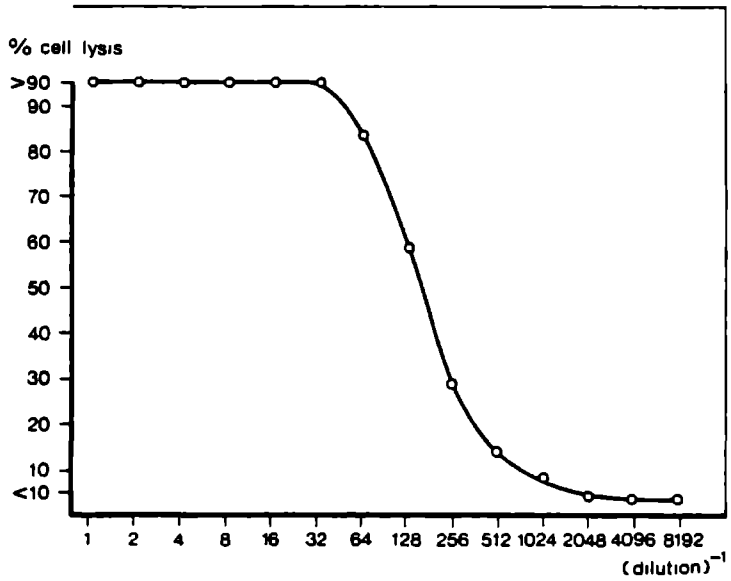

Fig. 2. Cytolysis of B10.D2 spleen cells by anti H-2K IgG2, in the presence of rabbit complement. The undiluted preparation contained 10 µg of IgG2/ml.

90% cytotoxicity is obtained. This is in contrast to the results with anti Ia sera, which usually react only with B cells that make up 45 % of the splenic lymphocyte population. The specificity of the anti-SD IgG2 was tested on C3H/He lymphoid cells. No cytotoxic activity towards these cells could be observed and this showed that the cytotoxicity of the anti SD IgG2 was directed specifically to H-2 antigens of the $H\text{-}2^{d}$ haplotype. The preparation can be used for intravenous injection into mice. We found that the removal of hemoglobin by washing before elution was a critical step in the procedure. If this was not done, the eluate contained large amounts of hemoglobin that were difficult to remove. The hemoglobin-containing eluate could not be used *in vivo* because, due to its toxicity, all mice died after intravenous injection. However, removal of hemoglobin and affinity chromatography on the anti-IgG2 column resulted in a non-toxic preparation. *In vivo* results obtained with this eluted fraction will be reported separately.

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Chapter 2

THE ROLE OF ANTI H-2K AND H-2D ALLOANTIBODIES IN ENHANCEMENT AND ACUTE ANTIBODY-MEDIATED REJECTION OF MOUSE SKIN ALLOGRAFTS

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Chapter 2 was accepted for publication in the Journal of Immunology

The enhancing and destructive effects of anti H-2K^{d} and anti H-2D^{k} antibodies (socalled SD antibodies) were studied in B6AF1 recipients of B10.D2 or B10.BR skin allografts. The SD antibodies were isolated from B6AF1 anti B10.D2 and B6AF1 anti B10.BR alloantiserum by adsorption to donor strain red blood cells and subsequent elution. Acute antibody-mediated rejection of B10.D2 or B10.BR skin grafts was readily induced by administration of anti H-2K or H-2D antibodies, respectively, along with rabbit complement.

Treatment of the B6AF1 recipients with the antibody eluates on day 0, 2, and 4 after transplantation resulted in specific and significant prolongation of graft survival.

Both graft destruction and enhancement can therefore be mediated by antibodies directed against the same SD specificity.

Alloantisera directed against products of the Major Histocompatibility Complex of the mouse (the H-2 complex) can influence the fate of organ or tissue allografts in two opposite ways. Destruction as well as protection of the graft can be induced by the same alloantiserum in a mouse skin transplantation model. Administration of B6AF1 ($H\text{-}2^{axb}$) anti B10.D2 ($H\text{-}2^{d}$) antiserum to B6AF1 recipients of B10.D2 skin grafts at days 0, 2, and 4 after transplantation prolongs graft survival from 10.4 to 18.9 days, whereas intravenous injection together with rabbit complement at day 7 or 10 in immunosuppressed recipients results in acute antibody-mediated rejection (AAR) of the graft (1), a phenomenon similar to hyperacute rejection in human renal transplantation. Attempts to separate the protective and destructive antibodies by physicochemical methods (2) or by isolation of pure subclasses of IgG (3) failed. It was shown by Staines et al. (4) that absorption of an enhancing mouse alloantiserum with donor strain erythrocytes yielded an antiserum directed against I region determinants which had unimpaired enhancing capacity. Jansen et al. (5) found that anti Ia IgG2, prepared by absorption of B6AF1 anti B10.D2 IgG2 with B10.D2 erythrocytes, was unable to cause AAR, whereas its enhancing capacity was equal to that of unabsorbed B6AF1 anti B10.D2 IgG2. It is obvious from these results that anti Ia antibodies play an important role in enhancement.

By these absorptions with B10.D2 red blood cells, antibodies directed against H-2K region products were removed along with the destructive activity, which suggests that an anti H-2K serum can cause AAR. To test this, the antibodies absorbed to the donor strain erythrocytes have to be eluted and purified. An isolation procedure for anti H-2K.31 eluates was described earlier (6). In this paper evidence is produced that by administration of H-2K as well as H-2D antibodies AAR can be induced. Using an anti H-2K or D antibody eluate, the role of these socalled SD (serologically defined) specificities in enhancement can be studied in the same transplantation model. In the literature contradictory reports can be found on the activity of anti SD sera in enhancement. In mouse skin allograft models, enhancement is difficult to achieve by administration of red blood cell eluates (7) or by anti SD sera raised in congenic recombinant mouse strain combinations (8). In other transplantation

models, however, enhancement can be induced with anti SD sera. This was reported for mouse tumor (9) and heart (10) allografts, and for rat kidney allografts (11). On the contrary, Catto et al. (12) could not induce enhancement of rat kidney allografts with an anti SD serum. Recently, McKearn et al. (13), using monoclonal anti Ag-B A antibodies, found preliminary evidence that these sera could induce enhancement of rat kidney allografts.

There can be no doubt that in several models enhancing activity of anti SD sera has been demonstrated. In mouse skin allograft models, however, only marginal prolongations have been reported (7, 8). In the current study, evidence is presented that administration of H-2K and H-2D antibody eluates caused significantly prolonged survival of mouse skin allografts, demonstrating that, apart from their destructive activity, anti SD antibodies can also induce immunologic enhancement.

MATERIALS AND METHODS

Animals

Inbred strains of B10.D2/new SN (H-2^d), A/HeJ (H-2^a), C3H/HeJ (H-2^k) and B10.BR (H-2^k) mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine). Balb/c (H-2^d) mice were obtained from the National Institutes of Health, Bethesda, Maryland. C57Bl/Rij (H-2^b) mice were obtained from the Radiobiological Institute TNO, Rijswijk, The Netherlands. (C57Bl/Rij x A/HeJ)F1 (=B6AF1) hybrids (H-2^{axb}) were raised by brother-sister matings.

Complement

Fresh frozen serum from New Zealand white rabbits was used as a source of complement. The rabbit sera were preselected, and only those sera that lysed 10% or less of mouse lymphocytes in the absence of allo-antibody were used.

Antisera

B6AF1 anti B10.D2 and B6AF1 anti B10.BR ascites fluids were raised as described (6). B6AF1 anti B10.D2 antibodies are directed against the H-$2K^d$ specificity 31 and the Ia^d specificities 11, 16 and 23. B6AF1 anti B10.BR is directed against H-$2D^k$ specificity 32; although there is a difference at the I-C region in this combination, no known anti Ia activity is present in this serum (14). Anti lymphocyte serum (ALS) was raised in goats by i.m. injections of C57Bl/Rij lymphocytes (15).

Analytical procedures

The amount of IgG2 in alloantisera and anti SD eluates was determined by radial immunodiffusion using anti IgG2 serum and mouse immunoglobulin standard from Meloy (Springfield, Va.). The amount of protein was quantitated by the method described by Lowry et al. (16).

Absorption procedure

Anti SD sera were prepared by absorption of complex alloantisera with pure red blood cells (RBC) carrying histocompability antigens of the donor haplotype according to a modification of the procedure described in a previous paper (6).
Anti H-2K.31 antibodies were prepared by absorption of B6AF1 anti B10.D2 serum with B10.D2 or Balb/C erythrocytes; anti H-2D.32 antibodies were prepared by absorption of B6AF1 anti B10.BR serum with B10.BR or C3H/HeJ RBC. Freshly drawn blood from 200 mice, collected in 8 ml portions in 3 ml anticoagulant citrate buffer, was centrifuged for 5 min at 2000 g at 4°C. The supernatant and buffy coat were discarded. The pelleted RBC were washed three times in phosphatebuffered saline (PBS) at pH 7.4; after each centrifuge run the buffy coat was carefully removed. Thereafter the RBC were purified further by passage through a closely packed cotton wool column (20 x 4 cm) as described by Diepenhorst et al. (17). In this way, more than 99% of leukocytes and platelets were removed. The purity of the RBC was controlled by examination of May-Grünwald-Giemsa stained smears of the column eluate. Only RBC suspensions containing less than 1 leukocyte per 10^5 erythrocytes were used. After passage through the column the RBC were washed once more with PBS, centrifuged at 200 g for 5 min, resuspended in the same volume of 0.125% glutaraldehyde in PBS, and incubated for 45 min at 4°C.
By this procedure, the cells are fixated, which reduces loss of antigen-antibody complexes during the subsequent incubation with alloantiserum, and cell lysis during the elution procedure. Glutaraldehyde was removed in two washings and the packed RBC were incubated overnight at 4°C in an equal volume of 2% (v/v) normal B6AF1 serum in PBS. In this way nonspecific absorption of protein during the subsequent incubation with antiserum was prevented. The RBC were then pelleted by centrifugation (5 min, 2000 g) and resuspended in an equal volume of 50% (v/v) of the appropriate alloantiserum in PBS.
Absorption was completed in 1 hr at room temperature under gentle shaking.

Elution procedure

After the absorption, the RBC were pelleted by centrifugation (5 min, 2000 g) and washed five times with five volumes of PBS. The RBC were then quickly suspended in five volumes of elution buffer, consisting of 1% (w/v) bovine serum albumine in 0.1 M glycine buffer at pH 2.7. The RBC were centrifuged at 2000 g for 5 min, the supernatant was taken and neutralized immediately to pH 7.4. with 2M Tris solution. A precipitate that formed during neutralization was removed by centrifugation (20 min. 2000 g).

Purification of eluted IgG

IgG was purified from the eluted fraction by absorption to protein-A-sepharose 4B (Pharmacia, Uppsala, Sweden). The eluate was absorbed batchwise overnight at 4°C. Elution was performed with 0.05 M citrate buffer at pH 2.6, containing 1 M NaCl. The protein-containing eluate was neutralized immediately with 2 M Tris solution, dialyzed overnight against PBS, concentrated to a volume of 10 ml by ultrafiltration and sterilized by passage through a sterile 0.2 µ membrane filter. The resulting anti SD preparations contained about 600 µg of protein per ml and 5-10 µg IgG2 per ml.

Cytotoxicity assay.

The *in vitro* cytotoxic activity of alloantisera and eluted fractions was determined in a lymphocytotoxicity assay as described (6).

Skin grafting

Transplantation of B10.D2 and B10.BR tail skin onto B6AF1 recipients was carried out as described earlier(15). The median survival time and the standard deviations were calculated according to the method of Litchfield (18).

Passive enhancement

Male B6AF1 recipients of a B10.D2 or B10.Br skin graft were treated on days 0, 2, and 4 after transplantation with 0.25 ml of whole alloantiserum or with different doses of anti SD eluate, injected i.p. Control animals received 0.25 ml of PBS.

Acute antibody-mediated rejection (AAR).

Female B10.D2 or B10.BR tail skin was grafted onto female B6AF1 recipients. The survival of these grafts was prolonged by the injection of 0.25 ml ALS, i.p., on days 0, 2, and 4 after transplantation. Rejection of the skingrafts caused by cellular mechanisms is postponed by this treatment to day 25-30 after grafting. On day 7, recipient mice were injected i.v. with different doses of anti SD eluate or alloantiserum along with rabbit complement. AAR was defined as complete necrosis of the skin graft, ocurring within 24 to 72 hr after i.v. injection.

RESULTS

The *in vitro* properties of the anti H-2K and H-2D preparations are shown in table I. In the eluates very low amounts of IgG2 are present, however with a high specific activity. Since IgG2 is the major complement-fixing subclass of IgG in the mouse, and therefore accounts for the greater part for the *in vitro* cytotoxic activity of the anti SD eluates, specific activity was expressed per µg of IgG2.
The *in vivo* activities of the anti H-2K and H-2D eluates in acute antibody-mediated rejection are shown in table II. AAR was readily induced both by i.v. injection of anti H-2K and H-2D antibodies in B6AF1 recipients of the appropriate skin allografts, along with rabbit complement. The table shows that by a dose as low as 1 µg of anti H-2K.31 IgG2, AAR of B10.D2 skin could be induced. In the control re recipients, treated with anti SD antibodies directed against the irrelevant specificity, no AAR was observed.

TABLE 1

In vitro properties of the anti H-2K and H-2D RBC eluates compared to the corresponding alloantisera

Source of antibody	Protein content (mg/ml)	Concentration of IgG2 (μg/ml)	Cytotoxic titer	Specific[a] activity	Purification[b] factor
B6AF1 anti B10.D2 serum	32.8	5300	1:128	24.2×10^{-3}	
Anti H-2K eluate	0.7	10	1:128	12.8	500
B6AF1 anti B10.BR serum	34.6	5000	1:32	6.2×10^{-3}	
Anti H-2D eluate	0.5	5	1:64	12.8	2,000

[a] Specific activity was defined as $(\text{cytotoxic titer})^{-1}$ per μg IgG2.

[b] Purification factor was defined as $\frac{\text{specific activity in the eluate}}{\text{specific activity in the whole alloantiserum}}$

TABLE II

Activity of the anti H-2K and H-2D eluates in acute antibody-mediated rejection of established skin grafts in B6AF1 recipients

Donor (haplotype)	Treatment[a]	Specificity recognized	Dose of IgG2 (μg)	No. of recipients	AAR
B10.D2 ($H\text{-}2^d$)	anti $H\text{-}2K^d$	31	5	3	+[b]
			4	1	+
			3	4	+
			2	4	+
			1	4	+
	anti $H\text{-}2D^k$	-	8	3	-
B10.BR ($H\text{-}2^k$)	anti $H\text{-}2D^k$	32	8	3	+
			5	2	+
	anti $H\text{-}2K^d$	-	5	5	-

[a] Recipients were injected i.v. on day 7 after transplantation with the eluate, along with 0.25 ml of rabbit serum, as a source of complement.

[b] + indicates complete necrosis of the skin graft within 48 to 72 hr after i.v. injection of the eluate plus rabbit complement.

TABLE III

Activity of the anti H-2K and H-2D eluates in enhancement of skin allografts in B6AF1 recipients

Donor (haplotype)	Treatment[a]	Dose of IgG2 (µg)	No. of recipients	MST[b]	SD[c]	p[d]
B10.D2 ($H\text{-}2^d$)	none	-	10	10.4	1.04	-
	PBS, 0.25mlx3		9	10.4	1.04	NS[e]
	anti H-2K, 0.25mlx3	2.5x3	10	12.4	1.06	<0.001
	anti H-2K, 0.40mlx3	4.0x3	13	14.4	1.05	<0.001
	B6AF1 anti B10.D2 serum, 0.10mlx3	500 x3	10	18.9	1.06	<0.001
	anti H-2D, 0.40mlx3	2.0x3	6	10.4	1.10	NS
B10.BR($H\text{-}2^k$)	none	-	10	14.7	1.22	-
	PBS, 0.25mlx3	-	10	15.1	1.14	NS
	anti H-2D, 0.40mlx3	2.0x3	5	16.9	1.10	<0.01
	B6AF1 anti B10.BR serum, 0.25mlx3	1250 x3	23	17.4	1.10	<0.001
	anti H-2K, 0.50mlx3	5.0x3	6	13.8	1.07	NS

[a] B6AF1 recipients were injected i.p. on days 0, 2, and 4 after transplantation.

[b] MST = median survival time.

[c] SD = standard deviation.

[d] p = level of significance (student's t-test) of the difference between the treated and the control group.

[e] NS = not significant.

The activity of the same anti SD preparations in enhancement is shown in table III. Treatment of B6AF1 recipients of a B10.D2 skin graft with 2.5 µg of the anti H-2K preparation on days 0, 2, and 4 after transplantation prolongs graft survival with two days, which is a significant prolongation in comparison with the untreated control group. A dose of 4.0 µg increased the anti H-2K induced prolongation up to 4 days. Administration to the recipients of 0.1 ml of unfractionated B6AF1 anti B10.D2 serum prolonged graft survival to 18.9 days. In the specificity controls, treated with 2.0 µg anti H-2D eluate, no enhancement was observed. In B6AF1 recipients of B10.BR skin grafts, treatment on days 0, 2, and 4 with 2.0 µg of anti H-2D resulted in significant prolongation of graft survival from 14.7 to 16.9 days.
Administration of 0.25 ml of whole B6AF1 anti B10.BR serum prolonged graft survival to 17.4 days. Treatment with 5.0 µg anti H-2K eluate as a specificity control did not induce enhancement in this model.

DISCUSSION

The results show that alloantibodies directed against H-2K and H-2D region determinants can cause destruction of mouse skin allografts. This is in agreement with the results of McKenzie and Henning (19) who used whole alloantisera in recombinant donor-recipient combinations in which only K or D region specificities of the donor were recognized by the antibodies. It is clear that acute antibody-mediated rejection is a function of SD antibodies.
Ia antibodies, even in high doses, are not capable of evoking acute rejection of mouse skin allografts. This was shown by Jansen et al.(5), using antisera shorn of SD antibodies by absorption to donor strain RBC, and confirmed later by McKenzie and Henning (19), using recombinant mouse strain combinations.
The different activities of anti SD and anti Ia antibodies in acute rejection can not be explained satisfactorily, since the presence of both SD and Ia antigens in skin tissue has been demonstrated by rejection of skin allografts in recombinant strain combinations (20). There are,

however, two possibilities to explain the difference in activity. The amount of Ia antigen in the graft can be too small to function as target for humoral attaque. Another explanation might be that the I region products causing cellular rejection of skin grafts are not identical with the serologically detectable Ia antigens. We consider the former explanation to be the more likely.

The results presented in this paper indicate that anti H-2K and H-2D antibodies, apart from their destructive capacities, can play a role in the protection of mouse skin allografts. Since the anti H-2K and H-2D antibodies were isolated by elution of absorbed antibodies from red blood cells, one should be sure that no contaminating anti Ia antibodies are present. Especially the enhancing activity of the preparations could be easily caused by small amounts of anti Ia. Sources of contaminating anti Ia are antibodies absorbed to leukocytes that are left in the donor strain erythrocyte suspensions, and IgG that is non-specifically absorbed onto the RBC. In our protocol, the erythrocyte suspensions were prepared by removal of the buffy coat in several centrifuge runs and passage through a cotton wool column. The purity of the suspensions was controlled by microscopic examination of stained smears of the cotton wool column eluates. Only suspensions containing less than 1 leukocyte per 10^5 erythrocytes were used. Assuming that the absorption capacity of leukocytes exceeds that of erythrocytes by a factor 50 (21), at most 0.05% of the antibody activity in the anti SD eluates could be attributable to Ia antibodies. In the dose of 4.0 µg anti H-2K eluate we used, this would be 2 ng. Taking into account the 500-fold purification, this corresponds with 1 µg of non-purified B6AF1 anti B10.D2 IgG2 with Ia specificity. From the results of Jansen et al. (3) we know that this is a factor 19 below the lowest enhancing dose of anti Ia IgG2 antibodies.

It is highly unlikely that non-specific absorption of serum proteins including immunoglobulins from the alloantiserum to the RBC caused substantial contamination with Ia antibodies. Assuming that a dose of 4 µg of anti H-2K IgG2 would merely consist of non-specifically absorbed IgG2, it would not suffice to account for the prolongation observed.

We have previously shown that at least 19 µg of B6AF1 anti B10.D2 IgG2 is required to obtain a comparable prolongation of graft survival (3). In B6AF1 recipients of B10.BR skin grafts, significant enhancement could be induced by administration of anti H-2D eluate. Contamination of this preparation by anti Ia antibodies is unlikely, because, although there is a difference in the I-C subregion in this combination, no serologically detectable anti Ia antibodies are raised (14). Administration of whole B6AF1 anti B10.BR serum resulted in prolongation of graft survival comparable to that induced by administration of the anti H-2D eluate. This indicates that during the absorption and elution procedure no enhancing activity, attributable to putative Ia antibodies, is lost. Administration of whole alloantiserum in the B10.D2 → B6AF1 model does induce significantly longer prolongation of graft survival than does administration of the anti H-2K eluate. Absorption to and elution from the donor strain RBC in this model accordingly results in loss of enhancing Ia antibodies.

It is not clear at which level in the immune response the enhancing antibodies exert their blocking functions. Ia antigens are thought to play an important role in the recognition phase of the immune response (22). A model of collaboration between Ia recognizing Ly 1^+ T-helper cells and K, D recognizing Ly $2,3^+$ T-killer cells was proposed (23), based primarily on *in vitro* results. This dichotomy concept was confirmed by the report of Nagy et al. (24), who found that Ly 1^+ T-cells specifically bound I-region products, and Ly $2,3^+$ T-cells bound K-region products. It was suggested that enhancement by anti Ia antibodies is caused by inhibition of the recognition of I-region antigens from the graft by Ly 1^+ T-helper cells (25). This was in line with the reported *in vitro* blocking of the mixed lymphocyte reaction by Ia antibodies (26), since this test can be regarded as an *in vitro* analogon of the *in vivo* recognition and proliferation phase.

In vivo, anti Ia antisera induced increased opsonization of antigen reactive-cells in the liver (27). These antigen-reactive cells expressed the Ly 1^+ phenotype (28).

Recent reports have shown, however, that K/D antigens on their own can give rise to an immune response. K as wel as D region differences can

lead to mixed lymphocyte culture stimulation, induce a graft versus host reaction, give a positive result in the cell-mediated lymphocytotoxicity assay, and can lead to rejection of heart, tumor and skin grafts (20, 8). It is conceivable that anti H-2K or H-2D antibodies can play a role in blocking of the recognition of K and D antigens, analogous to anti Ia blocking. Increased opsonization of antigen-reactive cells in mice treated with complexes of spleen cell antigen and anti H-2K.33 antibodies has recently been reported (28), indicating that anti H-2K antibodies do have activity at this level.

We conclude from our results that anti H-2K and H-2D antibodies have a weak but significant protective effect on graft survival in our model. The antibodies are probably working via the same mechanism as in enhancement by Ia antibodies, i.e. via the formation of immune complexes consisting of anti H-2K or D and K or D antigens that are released from the graft. Free antigenic determinants present in these complexes bind to antigen reactive cells and subsequently inactivate these cells by means of Fc-dependent mechanisms (29, 30). Opsonization of these cells is probably an important way by which this inactivation takes place (31).

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Chapter 3

IN VIVO ACTIVITY OF AN H-2 ALLOANTISERUM PURIFIED BY AFFINITY CHROMATOGRAPHY ON TRANSPLANTATION ANTIGENS

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Chapter 3 was published in Transplantation 1979
Vol. 27, No. 6, page 376

Transplantation antigens were isolated from murine tissue by solubilization with NP-40 and purification on a lentil lectin-sepharose column. The glycoproteins eluted from the lectin column were coupled to CNBr-activated Sepharose to prepare a specific immunoadsorbent. Intact histocompatibility antigens were demonstrated on the carrier particles in a cytotoxic inhibition assay with specific antiserum and spleen lymphocytes. Adsorption and subsequent elution of alloantiserum directed against the coupled antigens yielded a 30-fold purified product. The presence of both active K and I region products on the column was demonstrated by the *in vivo* activities of the alloantibodies eluted from the coupled molecules. Both acute rejection, a function of H-2K antibodies, and passive enhancement, a function of Ia antibodies, could be induced by administration of the eluate to recipients of skin allografts. These *in vivo* results show that the adsorbent can be used for preparative purposes.

Passive enhancement of allografts in the mouse can be brought about by the injection of antisera directed against I region products, and probably to a less extent by antisera against H-2K and D determinants (1). Enhancing Ia antisera can be raised in congenic mouse strain combinations that differ only in the I region of the H-2 complex. Alternatively, Ia antisera can be prepared from more complex antisera by absorptions with suspensions of red blood cells or platelets from appropriate mouse strains. By using this procedure contaminating antibodies against H-2K and D region antigens are removed (2,3). Although several authors have reported that with this method extensive prolongation of graft survival may be obtained in laboratory animals, it has the practical disadvantage that large amounts of cells of defined specificity are required for the absorptions.
In additon, the absorption of sera with live cells is complicated by the possible shedding of membrane constituents, frequently in the form of immune complexes. Zola (4) found that 11.9% of membrane-associated radioactivity was released from lymphoblastoid cells during a 30-min. absorption with antilymphocyte globulin at 4°C. Davies and Alkins (5) did not detect any immune complexes after RBC absorption, which suggests that the nature of absorbing cells is of importance in membrane shedding. Nevertheless, the possible release of immune complexes, which might be pathogenic, makes this approach less attractive for *in vivo* uses. A purification method, that gives rise to significantly less complexes makes use of specific immunoadsorbents prepared by the coupling of isolated antigen to a carrier particle. Several authors have used these columns for analytical purposes and have emphasized their reusability (6-8).
Depending on the nature of the coupled antigen, either the unbound fraction or the immunoglobulins eluted afterward may be usefull. To investigate whether products coded for by the K and I regions can be used for the production of immunadsorbents, a Sepharose column of CNBr-coupled glycoproteins, including transplantation antigens, was made for preparative purposes. A mouse alloantiserum containing antibodies against I en K region antigens was absorbed to this affinity column. The H-2K antibodies present in this serum evoke acute rejection of skin allografts, while the Ia antibodies give enhancement. The presence of both activities

in the antibody eluted from the column would indicate that K as well as I region antigens had been effectively coupled. Therefore, the eluate was administered to recipients of skin allografts and tested for the presence of destructive and enhancing activity.

MATERIALS AND METHODS

Animals

B10.D2/Sn (H-2^d) mice were originally obtained from The Jackson Laboratory, Bar Harbor, Maine. C57BL6/Rij (H-2^b) and A/Hej (H-2^a) mice were obtained from the Radiobiological Institute, Rijswijk, The Netherlands. B6AF1 hybrids were produced in the laboratory by mating of C57BL6/Rij and A/HeJ mice.

Antisera

Alloantisera were prepared by injecting female mice at weekly intervals, i.p., with approximately 5 x 10^7 allogeneic lymphoid cells in complete Freund's adjuvant. After five injections, most animals developed ascites and could be tapped every 1 to 2 weeks. The ascites fluid of several weeks was pooled and, before use, all antisera were heated at 56°C for 45 minutes and sterilized by passage through a sterile 0.2-μ filter. The cytotoxic titer of the ascites fluid was the same as the titer in the serum of the corresponding mice. Antilymphocyte serum was prepared by a s.c. injection of 5 x 10^7 C57BL/6 lymphoid cells in complete Freund's adjuvant into a goat.
This priming dose was followed by weekly i.v. boosters of the same number of C57BL/6 lymphoid cells. The animals were bled 1 week after the fourth immunization, the serum was heated at 56°C for 45 min, and absorbed once with C57BL/6 red blood cells (five volumes of serum/one volume of packed red blood cells). After absorption, this antilymphocyte serum was not toxic to the recipients if injected i.p.

Lectin isolation and coupling

Lens culinaris hemagglutinin (lentil lectin) was isolated from brown lentils according to the procedure described by Agrawal and Goldstein (9) for the isolation of concanavalin A. The lectin was purified by affinity chromatography to Sephadex G-150 (Pharmacia), using a 0.1 M

methyl-α-D-mannoside (Sigma) solution as eluent. Purified lectin was coupled to Sepharose-4B (Pharmacia) as described earlier for the coupling of IgG2 (10).

Protein estimation

Total protein was measured according to the method of Lowry et al. (10a) with the following modification. To estimate the amount of protein solubilized in 0.5% NP-40, a standard curve was similarly determined with bovine serum albumin in 0.5% NP-40 as a standard, and, before reading the test, a precipitate which formed during the incubation with phenol reagent was removed by centrifugation. The amount of IgG2 was determined with the radial immunodiffusion method of Mancini et al. (10b). One milliliter of agar gel was mixed at 56°C with 20 µl of specific goat antiserum, directed against murine IgG2 (Meloy).

Cytotoxicity assay

Cytotoxic tests were performed using the trypan blue dye exlcusion method as described earlier (11). Rabbit serum was used as a source of complement. Cytotoxic units are expressed as units per ml, i.e., cytotoxic titer^{-1}/ml. Thus, in a typical assay, using 0.1 ml of sample, the concentration of cytotoxic units is given by the inverse titer times 10.

Cytotoxic inhibition assay

Coupled antigen activity was determined in a two-stage inhibition test. A dilution series of antigen-Sepharose in Hanks' balanced salt solution (wherein undiluted = packed Sepharose) was preincubated at 37°C for 30 min with a fixed alloantiserum dilution, which gave 90% cytolysis in the control experiment. One hundred microliters of antigen-Sepharose dilution were mixed with 100 µl of antiserum dilution.
The Sepharose spheres were then carefully filtered away to prevent activation of the alternative pathway on the carbohydrate material and subsequent complement consumption (12).

One hundred microliters of the mixture were added to 100 µl of cell suspension (5 x 10^6 cells/ml) and 100 µl of rabbit complement. After an additional 30-min incubation at 37°C the test could be read. The inhibition titer is expressed as the dilution of antigen-Sepharose giving 50% inhibition of cell lysis.

In vivo studies

B10.D2 donor tail skin was grafted onto the right dorsal flank of a B6AF1 recipient. The graft was fixed with Nobecutane spray. A Band-aid was used as a cover. The donor and recipient mice were between 6 and 10 weeks old. The Band-aid was removed on day 6 after transplantation and the fate of the grafts followed by daily macroscopic inspection. Rejection was considered to be complete when no viable epidermis was left. Median survival time and SEM were calculated according to the method of Litchfield (13). Enhancement of skin grafts was induced by i.p. injections of 0.25 ml of alloantiserum at days 0, 2, and 4 after grafting (3). For acute antibody-mediated rejection (AAR), a previously described protocol was followed (14). Briefly, antilymphocyte serum-immunosuppressed recipients carrying established skin grafts received an i.v. injection of 0.25 ml of alloantiserum and 0.25 ml of rabbit complement at day 7 after transplantation. This resulted in destruction of the graft within 48 hr.

RESULTS

Antigen isolation and coupling

The procedure for the isolation and coupling of transplantation antigens was originally set up in the C57BL/6 (H-2^b) → B10.D2 (H-2^d) model. A 1% w/v homogenate was prepared in cold phosphate-buffered saline (PBS), pH 7.4, from C57BL/6 lymphoid tissue (spleen, thymus, and mesenteric lymph nodes) by treatment with a Potter-Elvehjem homogenizer. To this suspension, a 10% solution of NP-40 in PBS was added to a final concentration of 0.5%. The extraction mixture was stirred at 4°C for 30 min and the

solubilized fraction isolated by centrifigation for 30 min at 48,000Ig. This fraction was subjected to lentil lectin affinity chromatography. The bound material was eluted with 0.1 M α-*methyl*-mannoside solution in 0.5% NP-40-PBS. The yield of an elution run was determined by the Lowry procedure, and amounted 10 to 20 mg of (glyco)protein. To this solution, 1 ml of CNBr-activated Sepharose-4B per mg of glycoprotein was added. Coupling was completed overnight under gentle stirring at $4^{o}C$, after which the gel was washed once and inactivated in 1 M ethanolamine (pH 9.0) by gentle stirring for 4 hr. To remove NP-40 detergent, the Sepharose was carefully washed with cold PBS. Noncovalently bound proteins were removed by treatment with 1 M NaCl. In this way, 80% of the protein was coupled.

Antigen activity, coupled to Sepharose, was measured in the cytotoxic inhibition assay. Figure 1A gives the inhibitory capacity of a preparation of C57BL/6 antigen-Sepharose. The inhibition of the cytotoxicity of a B10.D2 anti-C57BL/6 serum against C57BL/6 lymphocytes was specific, since the cytotoxicity in an irrelevant system (B6AF1 anti-B10.D2 serum against B10.D2 lymphocytes) was unaffected by the immobilized antigen. Therefore, it seems that intact C57BL/6 antigen is present on the Sepharose spheres.

To test both the absorption-elution qualities of an antigen-Sepharose column and the *in vivo* activities of the eluate, the same procedure was carried out using B10.D2 tissue as the starting material. In this way, the *in vivo* testing can be performed in the B10.D2 ($H\text{-}2^{d}$) → B6AF1 ($H\text{-}2^{axb}$) model, in which the conditions for both acute antibody-mediated rejection and immunological enhancement of skin grafts are well defined (3).

The isolation and coupling procedure was applied to material derived from 150 B10.D2 spleens. Antigen activity, coupled to the Sepharose carrier was tested in the cytotoxic inhibition assay (fig. 1B). Again, antigen activity was detectable on the Sepharose. With this preparation absorption and elution experiments were performed with a total volume of B10.D2 immunoadsorbent of 10 ml.

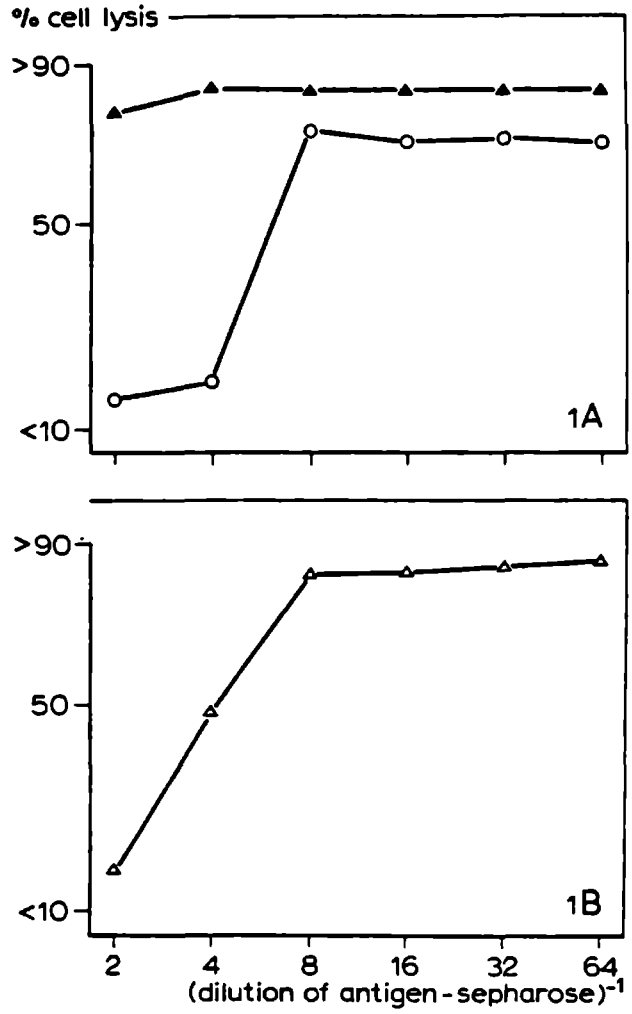

Fig. 1A. Inhibition of the cytotoxicity of B10.D2 anti-C57Bl/6 serum (1:6 dilution) against C57Bl/6 spleen cells (O) by immobilized C57Bl/6 glycoproteins. The cytotoxicity of B6AF1 anti-B10.D2 serum (1:32 diliution) against B10.D2 spleen cells (▲) was not inhibited.

1B. Inhibition of the cytotoxicity of B6AF1 anti-B10.D2 serum (dilution 1:64) against B10.D2 spleen cells (Δ) by immobilized B10.D2 glycoproteins.

Adsorption of B6AF1 anti-B10.D2 serum and elution from B10.D2 antigen-Sepharose

Five milliliters of B6AF1 anti-B10.D2 serum were applied to the antigen column. The column was washed with PBS at a flow rate of 300 ml/hr to remove unbound material. Adsorbed proteins were eluted with 0.05 M citrate buffer at pH 2.6, containing 1 M NaCl. The eluate was neutralized immediately with TrisHCL to avoid loss of biological activity. The column was reequilibrated with PBS. Eluates from two subsequent runs were mixed, concentrated by ultrafiltration to their original volume of 10 ml, dialysed against PBS, and sterilized by filtration through a 0.2-μ sterile membrane filter.

In vitro testing of the eluate

The amount of IgG2 in the eluate was determined by radial immunodiffusion. The IgG2 subclass is for the greater part responsible for the *in vivo* enhancing and destructive activities of the B6AF1 anti-B10.D2 serum (15). The activity of the eluate can therefore be expressed per μg of IgG2: this specific activity must increase during a successful purification procedure. The eluate contained 70 μg of IgG2 per ml. In a cytotoxic test against B10.D2 spleen lymphocytes, the cytolytic capacity of the eluate was determined. The result in Figure 2 shows that the cytotoxicity of the unfractionated B6AF1 anti-B10.D2 serum is almost completely preserved in the eluate. Specific activity, however, expressed as cytotoxic units per μg of IgG2, is raised by a factor of 30 (Table 1). Thus, absorption and elution of the B6AF1 alloantiserum on the B10.D2 spleen antigen column resulted in a purified antibody preparation, indicating again that specific antigen activity is preserved during the isolation and coupling procedure.

In vivo activity of the eluate

The unfractionated B6AF1 anti-B10.D2 serum is directed against H-2K and Ia determinants. It has two distinct activities in the B10.D2 → B6AF1

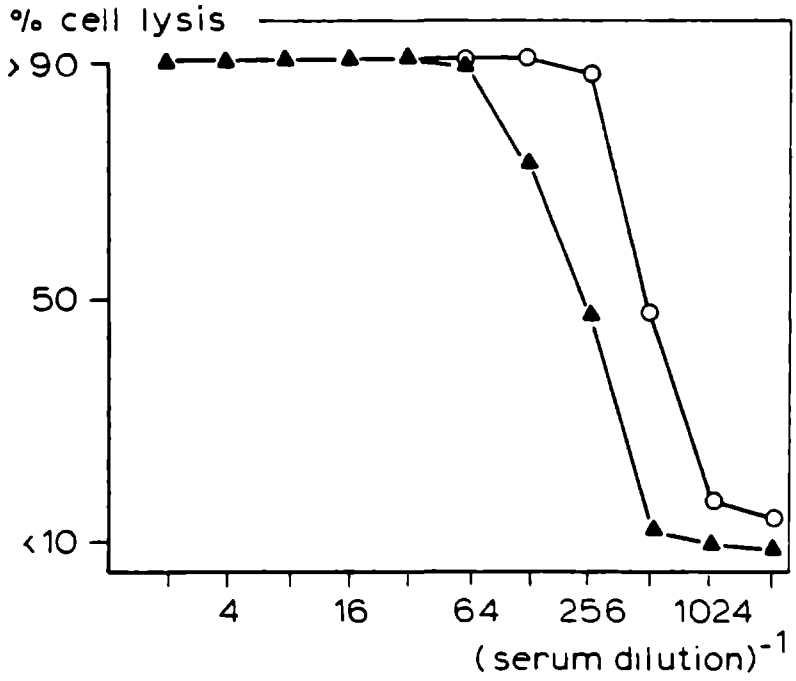

Fig. 2. Cytotoxicity against B10.D2 spleen cells of the antigen column eluate (▲) compared to whole B6AF1 anti-B10.D2 serum (O).

TABLE I

Specific activity of the antibody preparation

Sample	Concentration of IgG2 (μg/ml)	Cytotoxic units per ml	Specific activity
B6AF1 anti-B10.D2 serum	4000	5120	1.28
Antigen column eluate	70	2560	36.57

transplantation model. In the enhancement protocol, graft survival is prolonged from 11.2 to 18.9 days. The same antiserum induces graft destruction if injected along with rabbit complement at day 7 after transplantation. Antibodies directed against I region differences hace enhancing activity, while AAR cannot be evoked, not even with high dosages of Ia antibodies (3). On the other hand, the H-2K antibodies can cause AAR (unpublished observation), but their role in enhancement is not yet clear. So far, only marginal prolongation of graft survival has been obtained (1). Since both Ia and H-2K antigens have been subjected to the coupling procedure, we expected both activities to be present in the eluate.

Table 2 shows the activity of this preparation in the enhancement protocol. The enhancing activity is preserved in the column eluate. As in the cytotoxic test, the activity is somewhat less than that produced by the same volume of unfractionated alloantiserum, but the MST is still significantly prolonged as compared to that found with normal B6AF1 serum. Table 3 shows the activity of the eluate in the AAR protocol. Acute graft destruction results after the injection of the eluted antibody preparation together with rabbit complement. Here also, a higher dose is required to obtain the same effect as with whole antiserum.

DISCUSSION

The results show that a specific immunoadsorbent can be prepared by coupling NP-40-extracted and lectin-purified antigen to CNBr-activated Sepharose. The *in vivo* and *in vitro* properties of the absorbed antiserum are preserved in the eluted antibody population. The high purification factor, as demonstrated by the rise in specific activity after elution, is an indication that nonspecific binding of proteins to the immunoadsorbent is relatively low and that the activities of the eluate are therefore attributable to antibodies specifically bound to coupled antigen. In the literature eluates have been reported with 10 to 15 times increased specific activity *in vitro* (8,16).

The preservation of enhancing as well as AAR activity indicates that

TABLE II

Enhancing effect of the antibody preparation on B10.D2 skin grafts in B6AF1 recipients

No.[a]	Treatment[b]	MST[c] ± SEM *(days)*
10	3 x 0.25 ml of normal B6AF1 serum	11.2 ± 1.0
8	3 x 0.25 ml of column eluate	16.2 ± 1.1
10	3 x 0.25 ml of B6AF1 anti-B10.D2 serum	18.0 ± 1.1

[a]Number of recipients.

[b]Injection at days 0, 2, and 4 after grafting.

[c]MST, median survival time.

TABLE III

Destructive capacity of the antibody preparation on B10.D2 skin grafts in B6AF1 recipients

No.[a]	Treatment[b]	Acute antibody mediated rejection
10	0.25 ml of normal B6AF1 serum	-
10	0.25 ml of B6AF1 anti-B10.D2 serum	+[c]
7	0.5 ml of column eluate	+

[a]Number of recipients.

[b]Received injection of alloantiserum and 0.25 ml of rabbit complement at day 7 after grafting.

[c]+, rejection of the graft within 48 to 72 hr after injection.

both Ia and H-2K antigens are effectively coupled in this procedure. It should therefore be possible to prepare specific immunoadsorbents from isolated H-2K or D antigens with which polyspecific antisera can be absorbed to yield pure anti-Ia sera in such amounts, that they can be used *in vivo*. The main obstacle to this approach remains the source of pure antigen. Studies on the separation and purification of H-2K and D antigen via affinity chromatography on coupled antibodies have been carried out (17). It is possible that H-2K or D antigen will first have to be purified in such a way, since direct isolation from tissues like liver, which lacks Ia antigens, meets with serious difficulties (18, 19). Although the shedding of material from immunoadsorbents is much less than from viable or fixed cells, it is probably not completely absent (4). Better coupling methods will have to be designed to minimize this undesired effect. If these problems can be solved, the method may be very useful for the purification of defined antibodies from complex antisera.

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Chapter 4

PASSIVE ENHANCEMENT OF MOUSE SKIN ALLOGRAFTS BY ALLOANTIBODIES IS Fc DEPENDENT

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Chapter 4 was published in the Journal of Immunology 1979
Vol. 122, No. 2, page 421

The capacity of F(ab')2 fragments of alloantibodies to enhance mouse allografts was studied in B6AF1 recipients of B10.D2 skin grafts. F(ab')2 obtained by digestion of B6AF1 anti B10.D2 antibodies was purified by means of affinity chromatography, with anti-subclass antisera and protein A. The degree of contaminating IgG was less than 0.02%. Administration of F(ab')2 with an antigen-binding capacity similar to the IgG from which it originated, inhibited acute antibody-mediated graft rejection but was unable to induce enhancement. Even a dose that was 130 times the molar amount of the minimal enhancing dose of undigested IgG2 was ineffective. We conclude, therefore, that passive enhancement of mouse skin allografts by alloantibodies requires the Fc part.

Administration of alloantibodies to recipients of allografts can result in the enhancement of these grafts. The mechanism of immunologic enhancement is not fully understood and one important question is whether the Fc part of the alloantibodies is necessary to induce this phenomenon. F(ab')2 fragments lack cytotoxic activity and, if inducing enhancement, the use of F(ab')2 would overcome the problem of antibody-mediated rejection that can occur after administration of alloantibodies. The role of F(ab')2 has not only been studied in graft enhancement models, but also in systems where administration of antibodies induces specific suppression of antibody formation, a phenomenon that is probably related to enhancement. (Fab')2 could suppress the antibody response to sheep red blood cells *in vivo* (1,2) and *in vitro* (3,4), although IgG was much more suppressive than F(ab')2. In another study F(ab')2 was unable to suppress an *in vitro* response (5). Furthermore, F(ab')2 was unable to terminate an established immune response in contrast to IgG (6). In the mixed leukocyte reaction F(ab')2 fragments of histocompatibility antibodies were orginally shown to act inhibitory (7-9), but Nielsen et al. (10), using a more purified F(ab')2 preparation, found this inhibition to be Fc dependent. F(ab')2 fragments of alloantibodies induced enhancement of allografts of normal tissues (11-6) and also of tumors (17). When F(ab')2 was compared with IgG it was always found to be less active and this difference in activity suggested the presence of two mechanisms, one being Fc dependent and the other Fc independent. Crucial in these types of experiments is the purity of the F(ab')2 preparations used. Contaminating IgG might be responsible for some of the results obtained and might go undetected due to the relative insensitivity of the test systems used for IgG determinations. In the current study we have tried to exclude the role of contaminating IgG by using highly purified F(ab')2 fragments of alloantibodies. With such preparations we were unable to induce passive enhancement of mouse skin allografts in a model in which antibodies consisting of intact IgG induce significant prolongation of graft survival (18).

MATERIALS AND METHODS

Animals

Inbred strains of B10.D2 and B10.BR mice were originally obtained from Jackson Laboratory (Bar Harbor, Maine). B6AF1 hybrids were raised by mating of C57BL6/Rij and A/HeJ mice obtained from the Radiobiological Institute, Rijswijk, The Netherlands.

Antisera

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 (Difco, Detroit, Mich.). After 5 to 6 weeks the animals developed ascites, which was tapped weekly. The lymphocytotoxic activity of the ascites fluid was similar to the activity present in serum. The ascites fluid was sterilized by passing through a series of membrane filters (Schleicher and Schüll, Dassel, W. Germany) with decreasing pore sizes ranging from 8 µ to 0.2 µ. The preparation contained antibodies against antigens coded by the H-2K and I-regions of the mouse H-2 complex and against some minor non H-2 antigens (H-9). Anti-mouse F(ab')2 antibodies were raised in rabbits by intramuscular (i.m.) injection of 100 µg F(ab')2 fragments of mouse IgG in complete Freund's adjuvant. After three weeks, this was followed by weekly injections of 100 µg F(ab')2 in incomplete Freund's adjuvant. Anti-lymphocyte serum (ALS) was raised in goats by i.m. injections of 5 x 10^8 C57BL6/Rij lymphocytes suspended in complete Freund's adjuvant followed by two weekly i.v. injections of 5 x 10^8 C57BL6 lymphocytes in saline. Anti-mouse erythrocyte antibodies were removed from the ALS by absorption with C57BL6 erythrocytes. Goat antisera against mouse IgG and subclasses thereof, and against IgA and IgM, were obtained from Meloy Labs, (Springfield, Va). FITC conjugated horse anti-rabbit immunoglobulin serum was obtained from the Central Laboratory of the Red Cross Blood-transfusion Serive Amsterdam, The Netherlands. All antisera used *in vivo* were heat-inactivated at 56°C for 30 min.

Antibody columns

Sepharose 4B (Pharmacia, Uppsala, Sweden) was activated with 50 mg CNBr/ml (Koch Light, Colnbrook, U.K.) packed Sepharose at pH 10 according to Cuatrecasas (19). A 50% saturated ammonium sulphate precipitate of the various antisera was coupled after dialysis against phosphate-buffered saline (PBS) in a concentration of 5 mg/ml packed Sepharose overnight at 4°C. The degree of coupling was determined spectrophotometrically and ranged from 90 to 95%. The Sepharose was inactivated with 5 volumes 1 M ethanolamine (Merck, Darmstadt, W. Germany), pH 9.0, for 3 hr at room temperature and washed extensively with PBS.

Isolation of IgG

Antibodies from B6AF1 anti B10.D2 ascites fluid or normal B6AF1 serum were twice precipitated by addition of equal volumes saturated ammonium sulphate. After dialysis against 0.1 M Tris HCl buffer, pH 8.0, containing 0.2 M NaCl, the immunoglobulins were chromatographed on BioGel A 0.5 M, 200-400 mesh (Biorad, Richmond, Calif.). Instead of gel filtration Sepharose coupled Protein A (Pharmacia, Uppsala, Sweden) also was applied. IgG was eluted from this column with 0.1 M Na-citrate buffer, pH 2.8, containing 1 M NaCl. The eluate was immediately neutralized and dialyzed against PBS. Parts of these IgG preparations were used to prepare an IgG2 fraction by affinity chromatography as described earlier (18).

Isolation of F(ab')2

After dialysis against 0.1 M Na-acetate, pH 4.0, IgG (20 mg/ml) obtained from B6AF1 anti B10.D2 ascites fluid or normal B6AF1 serum was digested with 2% (w/w) pepsin (Boehringer, Mannheim, W. Germany) for 18 hr at 37°C. After adjustment of the pH to pH 8.0, the digest was chromatographed on BioGel A 0.5 M in order to remove pepsin and separate undigested IgG from F(ab')2. The pooled F(ab')2 fractions always contained undigested IgG as determined by polyacrylamide gel electrophoresis and by gel diffusion by using anti-mouse IgG subclass antisera that are only reactive with derminants present on the Fc part. F(ab')2 was further purified

either by rechromatography on BioGel A 0.5 M or by affinity chromatography with antibody columns or combinations of Sepharose-protein A and antibody columns. These columns were regenerated with one column volume of 0.1 M Na-citrate, pH 2.8, containing 1.0 M NaCl followed by extensive washing with PBS. To remove the final contaminants the preparations were batch-wise incubated with the antibody-coated Sepharose for 16 hr at room temperature.

Cytotoxicity assay

The *in vitro* cytotoxic activity of alloantisera was determined by trypan blue exclusion with B10.D2 or B10.BR spleen cells freed of erythrocytes by treatment with NH4CL. One-tenth milliliter of a cell suspension (5 x 10^6/ml) was incubated with 0.1 ml of a dilution series of alloantibody in Hanks' balanced salt solution and 0.1 ml of a dilution series of alloantibody in Hanks' balanced salt solution and 0.1 ml diluted (1:4) rabbit serum as 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.

Indirect immunofluorescence

One-tenth milliliter of B10.D2 or B10.BR thymocytes (5 x 10^6 cells/ml) was incubated with 0.1 ml of a dilution series of B6AF1 anti B10.D2 ascites fluid or F(ab')2 fragments thereof (5 mg F(ab')2/ml) for 30 min at room temperature. After three washings with PBS the cells were incubated with 0.1 ml of an appropriate dilution of rabbit anti-mouse F(ab')2 antiserum for 30 min at room temperature followed by three washings and a similar incubation with FITClabeled horse anti-rabbit immunoglobulin antiserum. After three washings the cells were scored for immunofluorescence. The whole procedure was carried out in the presence of 0.05% sodium azide to prevent capping.

Skin grafting

Transplantation of B10.D2 tail skin onto B6AF1 recipients was carried

out as earlier described (18).

Enhancement of allografts

If nor mentioned otherwise, enhancement was induced in B6AF1 recipients of B10.D2 skin grafts by i.p. injections of 0.25 ml alloantiserum or a F(ab')2 preparation on days 0, 2, and 4 after grafting.

Acute antibody-mediated graft rejection (AAR)

Acute rejection of well established grafts on immunosuppressed recipients could be achieved by i.v. injection of alloantibodies together with 0.25 ml rabbit C at day 10 after grafting (20). The recipients were immunosuppressed by i.p. injection 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 as measured in the *in vitro* test.

RESULTS

Purification of F(ab')2

Several steps were necessary to purify the F(ab')2 fragments of B6AF1 anti B10.D2 IgG. Undigested IgG was separated from F(ab')2 by gel filtration on BioGel A 0.5 M. The F(ab')2-containing fractions were pooled, concentrated and rechromatographed. Analysis of this F(ab')2 preparation on polyacrylamide gel electrophoresis showed a 7S band, and in gel diffusion with anti-mouse subclass antisera undigested IgG could also be detected. Therefore, anti-IgG1 and IgG2 antibody columns were applied twice to remove contaminating IgG. In such F(Ab')2 preparations small amounts of IgG and IgA could still be detected and therefore an isolation procedure was devised in which F(ab')2 was passed three times through a protein-A-column and four different antibody columns (IgG1, IgG2, IgM, IgA). After the final purification step F(ab')2 was concentrated

to 15 mg protein/ml and analyzed for contaminating Ig by radial immunodiffusion. No contaminating IgG1, IgG2, IgA or IgM could be detected anymore. With a standard of mouse immunoglobulins the lower limit of detection of the radial immunodiffusion technique was determined. The results of these isolation procedures are given in Table I. Analysis of the purest F(ab')2 preparation by polyacrylamide gel electrophoresis resulted in a single band that corresponded to a m.w. of about 115,000 dalton and that had a sedimentation coefficient of $S_w20 = 4.91 \pm 0.03$ as determined by analytical ultracentrifugation. Unless noted, this F(ab')2 preparation was used in all experiments.

In vitro activity of F(ab')2

In order to determine whether F(ab')2 had retained antigen-binding activity during the digestion or purification procedures a dilution series of F(ab')2 (5 mg/ml) was tested by indirect immunofluorescence and compared with the binding of B6AF1 anti B10.D2 ascites fluid, which contained 4 mg IgG/ml. Because anti-IgG antisera react predominantly with the Fc part of the IgG, rabbit anti-mouse F(ab')2 antiserum was used to obtain comparable results. The titers were 1:256 for F(ab')2 and 1:128 for the ascites fluid. Thus, F(ab')2 had similar antigen-binding activity as the original IgG, when compared on a molar basis. The titer with control B10.BR thymocytes was 1:2 both for F(ab')2 and IgG. The *in vitro* activity of F(ab')2 was also tested by the inhibition of the cytotoxic activity of IgG. Dilutions of B6AF1 anti B10.D2 and B6AF1 anti B10.BR ascites fluid giving 90% lysis, respectively, of B10.D2 and B10.BR spleen cells were used in the cytotoxicity assay after preincubation of the cells with a dilution series of F(ab')2 (5 mg/ml). The results given in Figure I show that F(ab')2 specifically inhibits the cytotoxic activity of B6AF1 anti B10.D2 alloantibody.

Inhibition of AAR of F(ab')2

Although antigen binding by F(ab')2 and blocking of cytotoxicity was shown *in vitro*, it is not self-evident that this also occurs *in vivo*.

TABLE I

Purification of F(ab')2 after pepsin digestion

Treatment	No. of Treatments	Percentage of contaminating[a] Ig
1. Bio-Gel A 0.5 M	2	7S Ig[b]
2. Bio-Gel A 0.5 M	1	IgA 3%
Anti-IgG1-Sepharose	2	IgG1 0.25%
Anti-IgG2[c]-Sepharose	2	IgG2 0.2%
3. Bio-Gel A 0.5 M	1	IgA < 0.03%
Protein A-Sepharose	3	IgM < 0.05%
Anti-IgG1-Sepharose	3	IgG1 < 0.02%
Anti-IgG2-Sepharose	3	IgG2 < 0.02%
Anti-IgA-Sepharose	3	
Anti-IgM-Sepharose	3	

a Determined by radial immunodiffusion with the use of F(ab')2 preparations with a concentration of 15 mg protein/ml.

b Determined by polyacrylamide gel electrophoresis.

c Antibody column contained anti-IgG2a as well as anti-IgG2b.

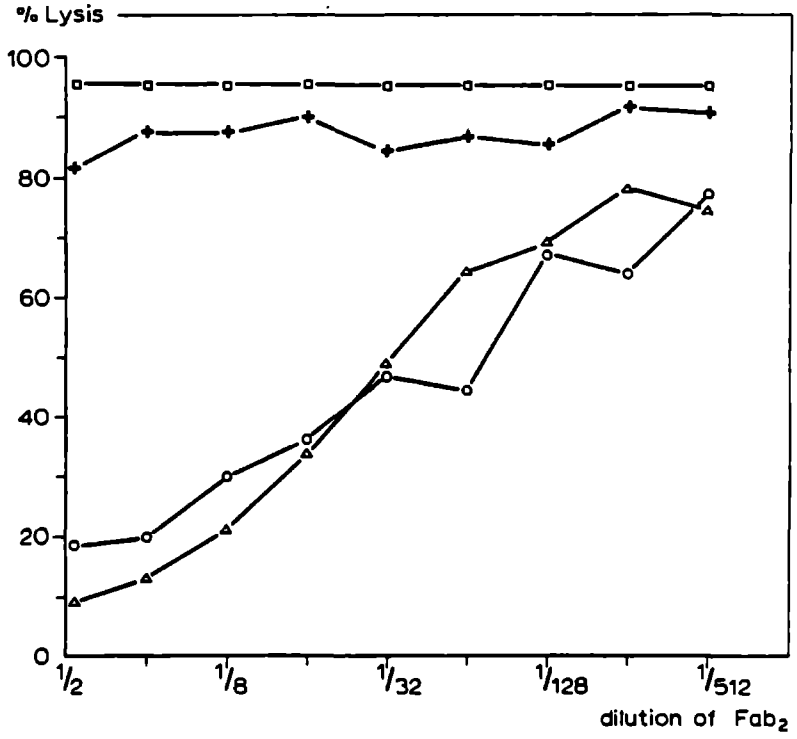

Fig. 1. Inhibition of cytotoxicity by B6AF1 anti-B10.D2 F(ab')2. The inhibitory capacity of a dilution series of F(ab')2 (5 mg/ml) was tested on a 1/150 dilution (Δ —— Δ) and a 1/200 dilution (O —— O) of B6AF1 anti-B10.D2 ascites fluid with B10.D2 spleen cells, or on a 1/6 dilution (□ —□) and a 1/8 dilution (+ —— +) of B6AF1 anti-B10.BR ascites fluid with B10.BR spleen cells (specificity-control).

The half-life of F(ab')2 *in vivo* is decreased in comparison with IgG (21). Whether F(ab')2 could sufficiently interact with the graft was tested by using the inhibition of AAR. Because AAR in mice is C-dependent and in this process the entire classical pathway is involved (22), F(ab')2 should be ineffective to induce AAR. This was tested by the administration of various amounts of F(ab')2, ranging from 50 to 1500 µg, together with 0.25 ml rabbit C, to immunosuppressed B6AF1 recipients of B10.D2 skin on day 10 after grafting. In none of the cases signs of rejection were observed whereas administration of IgG in this way always resulted in the acute rejection of the graft within 24 to 48 hr. The ability of F(ab')2 to inhibit this IgGinduced rejection was tested by the administration of 1 mg F(ab')2, 2 hr before the injection of 0.2 ml B6AF1 anti-B10.D2 antibodies and 0.25 ml rabbit C. As controls, either 0.2 ml alloantibody or 1 mg F(ab)2 was given together with rabbit C. The results given in Tabel II show that F(ab')2 inhibited the AAR completely, which proves that F(ab')2 can interact sufficiently long with the graft antigens to prevent the destructive effects of allo-antibody and C.

Enhancement of skin allografts by F(ab')2

The ability of F(ab')2 to induce passive enhancement of allografts was tested with two F(ab')2 preparations. One purified by rechromatography on Bio-Gel A 0.5 M and the second by gel filtration followed by extensive affinity chromatography. Both F(ab')2 preparations were noncytotoxic for B10.D2 lymphocytes and did not induce AAR. F(ab')2 purified by gel filtration was still contaminated with undigested IgG and F(ab'2) obtained after affinity chromatography contained less than 0.02% IgG (Table I). Three hundred-seventy-five micrograms contaminated F(ab')2, 500 µg pure F(ab')2 and 350 µg F(ab')2 from normal B6AF1 IgG were administered to three groups of B6AF1 mice at days 0, 2, and 4 after grafting of B10.D2 skin. Table III shows that pure B6AF1 anti-B10.D2 F(ab')2 did not enhance the graft whereas a significant increase in graft survival was obtained with F(ab')2 containing some contaminating IgG. The inability of F(ab')2 to induce enhancement might be due to the decreased half-life of F(ab')2

TABLE II

Inhibition of acute antibody-mediated graft rejection (AAR)[a] by F(ab')2

Treatment	No. Recipients	AAR	M.S.T. + S.D. *days*
None	8	-	28.0 ± 1.3
Alloantibody + complement	5	+	
F(ab')2 (1 mg) + alloantibody + complement	8	-	27.0 ± 1.1
F(ab')2 (1 mg) + complement	5	-	27.0 ± 1.0

AAR is evoked by administration of 0.2 ml B6AF1 anti-B10.D2 serum together with 0.25 ml rabbit complement to immunosuppressed B6AF1 recipients on day 10, after grafting of B10.D2 skin.

TABLE III

Passive enhancement by F(ab')2 in B6AF1 recipients of B10.D2 skin grafts

Treatment[a]	No. Recipients	M.S.T. + S.D.	p[b]
None	10	11.2 ± 1.0	
Normal B6AF1 F(ab')2, 500 µg	10	11.8 ± 2.0	N.S.[c]
F(ab')2 (contaminated with IgG) 375 µg	8	14.0 ± 1.2	<0.01
F(ab')2[d] (IgG < 0.02%), 500 µg	10	11.0 ± 1.1	N.S.

[a]Dose given on days 0, 2, and 4 after grafting.

[b]Level of significance (Student's *t*-test).

[c]N.S., not significant.

[d]F(ab')2 fragments of B6AF1 anti B10.D2 IgG.

in vivo. To exclude this possibility multiple daily injections were given. The experimental protocols and the results are given in Table IV. The administration of a total dose of 2500 µg F(ab')2 in this way did not induce enhancement. To compare the enhancing activity of IgG2 and F(ab')2 various amounts of B6AF1 anti-B10.D2 IgG2 and F(ab')2, ranging from 9 to 1875 µg, were given on days 0, 2, and 4 to B6AF1 recipients of a B10.D2 skin graft. The results given in Figure 2 show that three injections of 9 µg IgG2 already induced a significant enhancement, whereas F(ab')2 lacked effect in all doses tested.

DISCUSSION

Administration of F(ab')2 fragments of alloantibodies to recipients of mouse skin allografts did not result in the enhancement of the graft. This inability of F(ab')2 to induce enhancement is either due to a decreased capacity to bind antigen *in vivo* or to the absence of the Fc fragment. When tested in immunofluorescence F(ab')2 was as active as the IgG from which it was derived. In this test anti F(ab')2 antibodies were used in order to compare results obtained with F(ab')2 and IgG preparations. F(ab')2 was also able to inhibit specifically the *in vitro* cytotoxicity of alloantibodies. Thus, the antigen-binding capacity of F(ab')2, as measured *in vitro*, was not affected by the digestion or purification procedures. The effective binding to antigen *in vivo*, however, might be reduced by the decreased half-life of F(ab')2 (21). We tried to exclude this possibility by multiple daily injections of F(ab')2. Evidence that F(ab')2 could sufficiently interact with the graft is given by the inhibition of the acute antibody-mediated rejection. A single injection of 1 mg F(ab')2 completely inhibited the acute rejection by alloantibody. When comparing the enhancing activity of IgG2 and F(ab')2 we found that a total of 27 µg IgG2 already induced a significant enhancement, whereas a total of 2500 µg F(ab')2 had no effect. Compared on a molar base, IgG2 is at least 130 times more efficient than F(ab')2. From these findings we conclude that F(ab')2 fragments of alloantibodies are unable to induce enhancement of allografts. This is in contrast to findings reported

TABLE IV

Passive enhancement by multiple injections of F(ab')2 in B6AF1 recipients of B10.D2 skingrafts

Treatment	No. Recipients	M.S.T. ± S.D. *days*
Normal B6AF1 F(ab')2 350 µg on days 0, 2, and 4	10	11.8 ± 2.0
B6AF1 anti-B10.D2 F(ab')2 3 x 50 µg on days 0, 1, 2, and 3	10	11.2 ± 1.0
B6AF1 anti-B10.D2 F(ab')2 3 x 250 µg on day 0 2 x 250 µg on days 1, 2, and 3 1 x 250 µg on day 4	7	11.5 ± 1.2

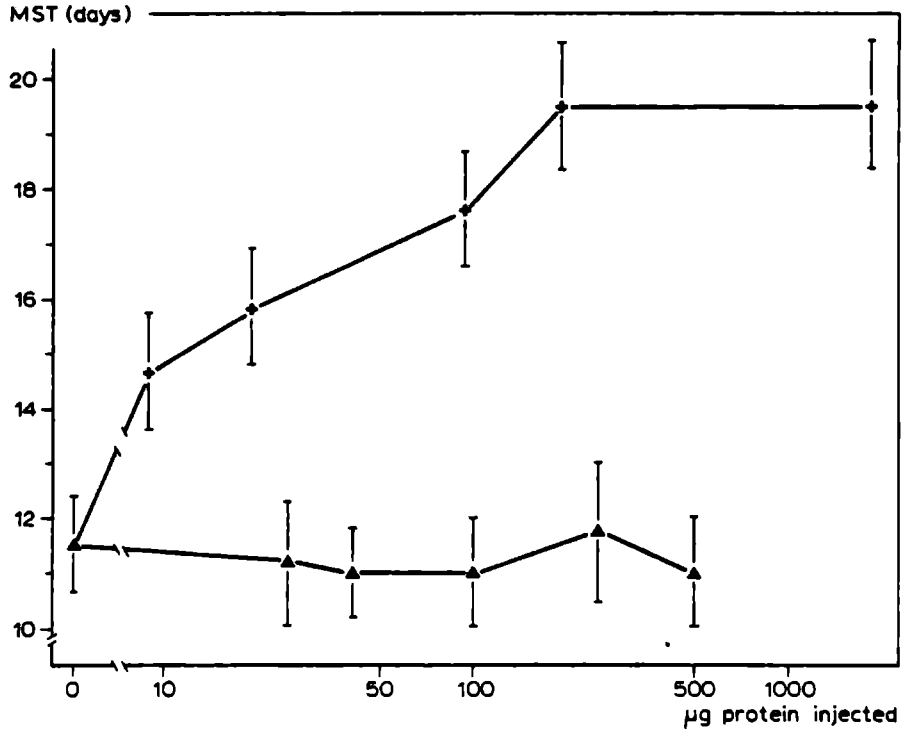

Fig. 2. Passive enhancement of B10.D2 skin grafts in B6AF1 recipients by administration of B6AF1 anti-B10.D2 IgG2 (+ —— +) or F(ab')2 fragments thereof (▲ —— ▲) on days 0, 2, and 4 after grafting.

by other authors (11-17). This might be due to differences in the purity of the F(ab')2 preparations used. In some studies enhancement was obtained by using preparations from which undigested IgG was not separated from F(ab')2 (11-14). In other reports F(ab')2 was separated from undigested IgG on the basis of differences in m.w. (15-17). In our hands such procedures resulted in an enrichment of F(ab')2 preparations but not in a total removal of undigested IgG. We could demonstrate that the amount of contaminating IgG remaining after rechromatography on Bio-Gel A 0.5 M was sufficient to induce significant enhancement. This enhancing activity could be removed by repeated affinity chromatography with protein A and anti-subclass antisera, which all react with the Fc part of immunoglobulins. The absence of cytotoxic activity is not a reliable measure of the degree of undigested IgG in a F(ab')2 preparation because of the inhibition of this cytotoxicity by F(ab')2 itself. We therefore used the reaction with anti-subclass antisera as a criterion for purity. The inability of F(ab')2 to induce enhancement shows that simple masking of antigenic determinants, although this occurs as is shown by the inhibition of AAR by F(ab')2, does not adequately explain the phenomenon of immunologic enhancement. A Fc-dependent mechanism should be at work.

A possible mechanism of enhancement could be that the administered antibodies form immune complexes with cellbound (passenger cells) or soluble antigens released from the graft. These immune complexes could then inactivate antigen reactive cells upon binding to these cells and the inactivation takes place by virtue of the Fc part. That this process indeed might occur is suggested by the opsonization of antigen reactive cells in rats bearing passively enhanced renal allografts (23,24). The inactivation mechanism does not necessarily occur via opsonization alone, but antibody-mediated cell-dependent cytotoxicity or C activation could also be involved. Apart from these theoretical considerations the results of our study suggest that the outlook for the use of F(ab')2 fragments to induce specific immunosuppression in clinical transplantation is not favorable.

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CHAPTER 5

PASSIVE ENHANCEMENT OF MOUSE TUMOR ALLOGRAFTS BY ALLOANTIBODIES IS Fc-DEPENDENT

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Chapter 5 was published in the Journal of Immunology 1979
Vol. 123, page 1353

Enhancement of growth of a B10.D2 fibrosarcoma in B6AF1 recipients could be induced by administration of B6AF1 anti B10.D2 lymphocyte serum. The role of the Fc part in this phenomenon was studied by treatment of the B6AF1 recipients with F(ab')2 fragments of enhancing alloantibodies. A highly purified F(ab')2 preparation was used to exclude any effects of undigested IgG. Administration of F(ab')2 did not lead to enhancement of the tumor allografts, not even when given in a dose that was 22 times the molar amount of the lowest enhancing dose of undigested IgG. We therefore conclude that passive enhancement of mouse tumor allografts by alloantibodies is Fc dependent.

Tumor allografts show enhanced growth in several animal models if the tumor-bearing recipients are injected with alloantisera against donor transplantation antigens. This phenomenon is caused by a specific antibody mediated suppression of the cellular immune response to the graft. Similarly, these antibodies can specifically inhibit cellular immune responses *in vitro*. Studies of the role of the Fc part of the antibody molecule in these reactions by using F(ab')2 fragments suggested that the inhibition was Fc-independent, both *in vitro* (13) and *in vivo* (4-8), including the inhibition of the cellular immune response against tumor allografts (9-11). These findings, however, are unexpected in the light of other studies demonstrating that the Fc part is required for feedback suppression of the humoral response by antibody *in vitro* (12, 13) and *in vivo* (14-16). Contaminating IgG in the F(ab')2 preparations might account for the suppressive activity on the cellular immune response. In earlier studies we have found that minimal amounts of IgG can induce specific immunosuppression leading to enhancement of mouse skin allografts (17). The purity of the F(ab')2 preparations used should therefore be well defined to exclude that intact IgG is still present in a suppressive dose. We now report the effect of highly purified F(ab')2 fragments of alloantibodies on tumor allograft survival, showing that even with high doses of F(ab')2 no enhancement can be induced in a model in which intact alloantibodies cause progressive tumor growth.

MATERIALS EN METHODS

Animals

Inbred strains of B10.D2/new SN(H-2^d), A/HeJ(H-2^a) and B10.BR (H-2^k) mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine, U.S.A.).
C57Bl6/Rij (H-2^b) mice were obtained from the Radiobiological Institute TNO, Rijswijk, The Netherlands. (C57Bl6Rij x A/HeJ)F1 (=B6AF1) hybrids (H-2^{axb}) were raised by brother-sister matings.

Antisera

B6AF1 anti B10.D2 and B6AF1 anti B10.BR ascites fluids were raised as described (18).

Preparation of F(ab')2 fragments

IgG was prepared from B6AF1 anti B10.D2 ascites fluid by adsorption on protein A, coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden). Elution was performed using 0.1 M Na-citrate buffer, PH 2.8, containing 1 M NaCl. The eluate was immediately neutralized and dialyzed against PBS. F(ab')2 fragments of B6AF1 anti B10.D2 IgG were prepared, purified and assessed for antigen binding activity according to the procedure described in detail by Capel et al (19).
Briefly, a pepsin digest of IgG was purified by affinity chromatography to protein A-sepharose and to immunoabsorbents consisting of goat antibodies against murine IgG1, IgG2, IgA and IgM (obtained from Meloy, Springfield, U.S.A.).
The amounts of intact antibody molecules in the final F(ab')2 preparation amounted to: IgA < 0.03%; IgM < 0.05%; IgG1 < 0.02% and IgG2 < 0.02%, as was determined by radial immunodiffusion.
The amount of protein in the IgG and F(ab')2 preparations was quantitated by the method described by Lowry et al. (20). The antigen-binding activity of the F(ab')2 molecules was determined by indirect immunofluorescence

and equalled that of intact IgG. In addition, it was shown that F(ab')2 was able to inhibit specifically the complement-mediated cytolysis of B10.D2 spleen cells by intact IgG.

Tumor transplantation

The tumor in this study was a mouse fibrosarcoma which was induced in a B10.D2 female mouse by a subcutaneous injection of 0.05 ml olive oil containing 1 mg 3-methylcholanthrene. The sarcoma had been transferred *in vivo* for three years at the time that the experiments were carried out. It is a slowly growing tumor, showing microscopically only few mitoses per field (magnification 400 x). The average tumor diameter, measured two months after intracutaneous injection of 4×10^6 cells in 20 syngeneic recipients, is 16.7 mm. To prepare a fibrosarcoma cell suspension, one tumor-carrying B10.D2 mouse was sacrificed and the tumor was taken out. The tissue was minced in Ringer's salt solution at $4^{\circ}C$ and suspended by gently pressing through a fine wire mesh. This suspension was left standing for 5 minutes and the supernatant was taken as free cell suspension. The cells were pelleted at 250 g for 5 minutes and resuspended in Ringer's solution. A sample was stained with Sedicolor (Molter GmbH Heidelberg, W. Germany) and tumor cells were counted in a Bürker counting chamber. The cell suspension was then diluted to a concentration of 2.5×10^7 cells/ml. Aliquots of this suspension containing 2×10^6 tumor cells were injected intracutaneously on the right dorsal flank of B6AF1 female recipients. Tumor growth was followed macroscopically by measurement with calipers during 3 months.

RESULTS

Tumor enhancement

Enhancement was induced in female B6AF1 recipients of B10.D2 sarcoma grafts by intraperitoneal injections of alloantiserum on days 0, 2, and

4 after transplantation. In this model no macroscopically detectable tumor growth is seen unless the recipients are treated with an enhancing antiserum. It is shown in Table I that only recipients treated with specific anti B10.D2 serum display visible tumor growth in most cases. The enhanced tumors grow progressively (fig. 1): the diameters of the tumors increase untill the recipients succumb. In the recipients not treated with with specific enhancing antiserum no tumor growth is observed, not even temporarily.

In vivo activity of F(ab')2

In this model the *in vivo* activity of F(ab')2 was tested. To be albe to compare the activity of F(ab')2 with IgG we first carried out dose-response studies with intact IgG. The results in Table 2 show that the threshold dose for induction of enhancement by IgG in this system lies between 300 and 960 µg. F(ab')2 is a dose of 2200 µg, comparable on a molar base to 2700 µg of IgG, was ineffective. The inability of F(ab')2 to induce enhancement might be due to the decreased half-life of F(ab')2 *in vivo* (21).
To exclude this, multiple daily injections were given. The experimental protocol and the results are also given in Table 2. The administration of a total dose of 17.0 mg (Fab')2 in this way did not induce enhancement of tumor growth, while 0.96 mg of IgG induced enhancement in 9 out of 10 animals, resulting in progressive tumor growth. The total amount of 17 mg of (F(ab')2 did contain at most 6.8 µg of contaminating IgG, which is a factor 44 under a non-effective dose of IgG (i.e. 300 µg).

DISCUSSION

In the tumor transplantation model which was developed the enhancing activity of antibodies and antibody fragments can be dertermined in a clear-cut way, since the effect is an all-or-none phenomenon. In recipients that were treated with control sera or non-enhancing fractions, no visible or palpable tumors developed. The tumor cells were readily

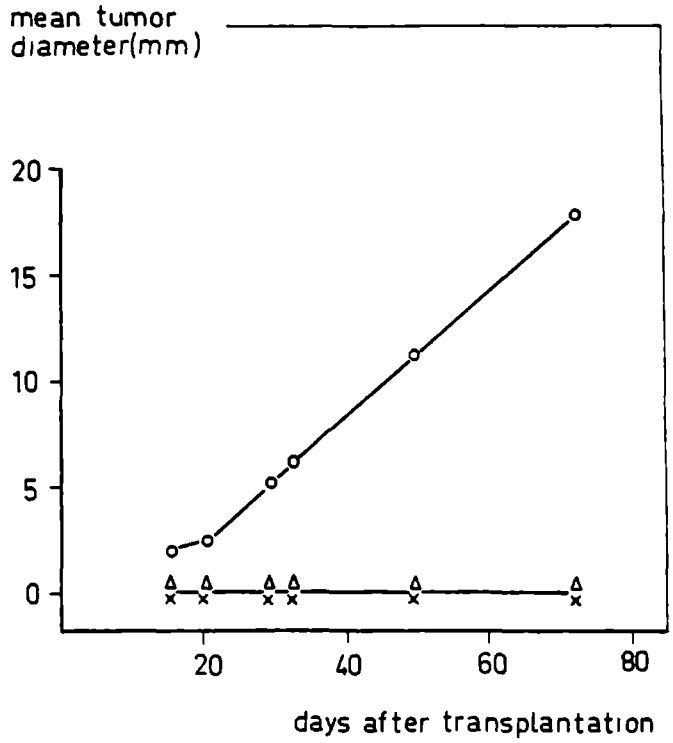

Fig. 1. Passive enhancement of B10.D2 fibrosarcoma grafts in B6AF1 recipients by administration of B6AF1 anti B10.D2 serum. Recipients were treated with 0.25 ml of B6AF1 anti B10.D2 serum (0 —— 0), 0.25 ml of B6AF1 anti B10.BR serum (Δ —— Δ), or 0.25 ml of normal B6AF1 serum (x —— x) at days 0, 2, and 4 after transplantation. Each point represents the mean value of tumor diameters in 5 animals.

TABLE I

Passive enhancement of B10.D2 fibrosarcoma grafts in B6AF1 recipients by administration of B6AF1 anti B10.D2 serum

Treatment[a]	No. of mice with growing tumors/total number tested
None	0/9
Normal B6AF1 serum	0/9
B6AF1 anti B10.BR serum	0/9
B6AF1 anti B10.D2 serum	18/25

[a] B6AF1 recipients were injected i.p. with 0.25 ml of serum on days 0, 2, and 4 after transplantation.

TABLE II

Influence of B6AF1 anti B10.D2 IgG and F(ab')2 administration on the growth of B10.D2 fibrosarcoma grafts in B6AF1 recipients

Treatment[a]	No. of mice with growing tumors/total number tested
100 µg IgG x 3	0/8
320 µg IgG x 3	9/10
900 µg IgG x 3	9/10
3000 µg IgG x 3	10/10
720 µg F(ab')2	0/17
1000 µg F(ab')2 x 2 on day 0 x 3 on day 1, 2, 3, 4 and 5[b]	0/10

[a] Unless stated otherwise, B6AF1 recipients were injected i.p. on day 0, 2, and 4 after transplantation.

[b] Injections were given with intervals of 6 or 9 hr.

accessible for the mediators of cellular immunity, as the inoculate consisted of a free cell suspension. Presumably tumor growth is too slow to form a solid tumor and to overrule in this way the host defense mechanisms for even a short period. Inherent to such a system is the relatively high dosis of antibody needed to give an enhancing effect. In our model the threshold dose lies between 300 and 960 µg, which is at least a factor 5 above the threshold dose in a skin allograft model (17). To test the enhancing activity of F(ab')2 in this model, a high dose therefore should be administered. A dose of 17.0 mg of F(ab')2, however, given in multiple daily injections over a period of 6 days, was ineffective (Table 2). This dose exceeds on a molar base the lowest enhancing dose of intact IgG (i.e. 960 µg) by a factor 22.

Our data indicate that administration of F(ab')2 fragments of alloantibodies to recipients of mouse tumor allografts does not result in enhancement of tumor growth. This finding is contrary to the results of others and we believe that contaminations of undigested IgG might explain some of the reported results. To exclude any enhancement effects of intact IgG, we used a highly purified F(ab')2 preparation in which the amount of undigested IgG was negligible.

It should be noted that the enhancing alloantiserum was raised with normal donor strain lymphocytes. It contained antibodies against the K and I region determinants of the major histocompatibility complex (MHC). The role of antibodies against tumor-specific antigens in this system will be the subject of further investigation. To examine the effects of such antibodies, an anti-tumor serum must be raised in a syngeneic model.

We conclude from our results that the Fc part of the alloantibody molecule is required for the induction of enhancement of mouse tumor allografts. This is in agreement with the results that we obtained in a mouse skin graft model (19), using a similarly isolated and purified F(ab')2 preparation.

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SUMMARY

Antibodies can influence the fate of transplanted organs or tissues in two opposite ways. Graft destruction mediated by antibodies has been reported to occur in experimental as well as in clinical transplantation. On the other hand, specific suppression of the immune response, leading to significant prolongation of graft survival, can be induced by administration of antibodies. This protective effect of antibodies, called passive enhancement, has thusfar been observed only in experimental animal models.

The aim of this study was to determine the experimental conditions that favour the induction of either phenomenon.
We approached this problem in a mouse skin allograft model in which both antibody-mediated graft protection and destruction could be reproducibly induced, and in a mouse tumor allograft model, in which graft protection led to progressive tumor growth.

In the first set of experiments we focussed on the specificity of the antibodies. In the antisera used, antibodies against K-, D-, and I-region products are present. It was shown in a previous study that anti Ia antibodies could induce enhancement but lacked any destructive activity. The role of anti H-2K and H-2D antibodies in these phenomena was not clear. We isolated these antibodies by absorption to and elution from donor strain red blood cells and tested their *in vitro* and *in vivo* activities. It was shown that highly purified anti H-2K or anti H-2D antibody fractions could be prepared in this way, which were suited for *in vivo* use. Acute antibody-mediated rejection could readily be evoked by administration of both anti H-2K and H-2D antibodies. The results showed in addition, that these anti SD (serologically defined) antibodies were able to induce enhancement, leading to significant and specific prolongation of skin allograft survival. On the basis of this latter finding we assumed that anti H-2K or H-2D antibodies could block the immune response, similarly to the blocking by Ia antibodies. This blocking of the response to H-2K or H-2D region antigens is in agreement

with the recent findings that K, D antigens on their own can give rise to an immune response without needing the help of antigenic differences in the I-region. Our results argue strongly against the earlier hypothesis that only I-region antigens are able to trigger the recognition and proliferation phase of the immune response, and that K- and D-region products serve only as the targets for the immune attack in the killing phase.

The eventual clinical application of enhancing antibodies will depend on the availability of large amounts of antibodies of defined specificity. A way to prepare such antibody fractions could be provided by affinity chromatography. The antigen against which the antibodies are directed is immobilized by chemical coupling to a carrier particle. In our experiments we tried to isolate the transplantation antigens against which the B6AF1 anti B10.D2 alloantiserum is directed by solubilization with NP-40 detergent from B10.D2 lymphoid cells. The antigens were partly purified and coupled to CNBr-activated Sepharose.
By absorption and elution of the alloantiserum, a 30-fold purified antibody preparation could be obtained, in which the *in vitro* and *in vivo* activities were preserved. The results show that, basically, this method can be used to isolate antibody fractions from complex sera. However, its usefulness largely depends on the feasibility to isolate K, D, or Ia antigens separately from cell surfaces.
The recently developed myeloma cell-fusion technique should be mentioned in this context. Probably this technique will provide a powerful tool in the preparation of large amounts of antibodies of defined specificity.

In earlier reports in the literature it was stated that it should be possible to induce enhancement with F(ab')2 fragments of allo-IgG. These F(ab')2 fragments lack the Fc part and are therefore not able to bind C1q and activate the classical pathway of the complement system. Preparation of F(ab')2 fragments would consequently provide one with an enhancing preparation that is not capable of inducing acute rejection. We prepared these F(ab')2 fragments from B6AF1 anti B10.D2 IgG and tested their *in vitro* and *in vivo* activities. The results showed that

the antigen-binding capacity of F(ab')2 equalled that of undigested IgG; the *in vitro* complement-dependent cytotoxicity of IgG could be inhibited with F(ab')2 fragments, that were non-cytotoxic themselves.
F(ab')2 also lacked *in vivo* cytotoxicity: acute rejection of skin allografts could not be induced by administration of F(ab')2. It was shown in addition that it was possible to inhibit the destructive activity of intact IgG *in vivo* by prior administration of F(ab')2 to the recipients of skin allografts.
We were, however, unable to induce prolongation of skin allograft survival or protection of tumor allografts by administration of F(ab')2 fragments, even when given in high doses over a number of days. Crucial in these experiments was the purity of the F(ab')2 fragments used, since it was known from earlier studies that minimal amounts of intact IgG can induce significant graft protection, resulting in prolonged survival of skin grafts.
It is clear from these findings that the possible applications of F(ab')2 fragments in clinical transplantation are drastically diminished. The absolute requirement of the Fc fragment for the induction of enhancement, that was demonstrated in two different transplantation models, threw new light, however, on the mechanism behind this phenomenon. We suppose that the Fc dependency of enhancement can be explained best by assuming that the enhancing antibodies form immune complexes with antigens released from the graft, and that these complexes bind subsequently to the antigen-recognizing cells. These cells will then be eliminated by Fc dependent mechanisms. It was actually shown by others that antigen-antibody complexes induced an increase in opsonization of antigen-reactive lymphoid cells in the liver of rats. These results show that lymphoid cells that have a central place in the recognition phase of the immune response can be effectively inactivated by specific immune complexes and this might well constitute the most important mechanism in enhancement.

Despite intensive investigations there has been made only little progress towards the application of enhancement as a tool in clinical immunosuppression. There is no firm evidence thusfar that enhancement can be induced in man: human recipients of kidney allografts with a positive B-

lymphocyte cross-match do not display significantly higher long-term survival rates than patients with a negative B-cell cross-match. There is, on the other hand, no proof that human B-cell antibodies, as they are measured in the cross-matching procedure, are comparable to the protective Ia-antibodies in the murine transplantation models, and it is therefore not justified to conclude from these observations that enhancement plays no role in clinical transplantation. It remains important to investigate the eventual clinical relevance of this form of specific immunosuppression, since enhancement would be very advantageous in human transplantation. The currently used immunosuppression depends mainly on steroid hormones, antimetabolite drugs, and anti-lymphocyte globulin, all of which induce nonspecific suppression of the immune response. The major risk of this type of suppression is infection with bacterial, viral, fungal or protozoan organisms.
A second harmful side-effect is a ten- to twentyfold increased risk of spontaneous malignant tumors. Enhancing antibodies do not have these draw-backs, since they exert their function specifically in combination with the donor transplantation antigens, and do not cripple the whole immune apparatus.

In the immune response to alloantigens there is apparently an intensive cooperation between cells and antibodies. This response is subject to several regulatory mechanisms, of which antibody-dependent suppression of cellular immunity is only one. Further investigation of this complex defense system holds the promise of finding a way of specific immunosuppression that would ultimately lead to improved graft prognosis in human transplantation.

CURRICULUM VITAE

Robert Marius Walther de Waal is geboren op 30 augustus 1951 te Dongen. Hij bezocht het Stedelijk Gymnasium te Nijmegen en behaalde het eind-diploma gymnasium B in 1969.
Vanaf dat jaar studeerde hij scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen in de richting S2 werd in 1973 behaald; het doctoraalexamen, met als hoofdrichtingen farmacochemie (prof. van Rossum) en biochemie (prof. Bloemendal) en als caputtentamen Instrumentele Methoden (dr. Hilbers) in 1975.
Vanaf augustus 1975 was hij als wetenschappelijk medewerker werkzaam aan het laboratorium van de afdeling Nierziekten (hoofd: prof. Wijdeveld) van de Universiteitskliniek voor Inwendige Ziekten (hoofd: prof. Majoor) te Nijmegen, waar de onderhavige studie werd aangevangen. Vanaf september 1977 is hij als wetenschappelijk medewerker werkzaam aan de afdeling Pathologische Anatomie (hoofd: prof. Vooys), waar hij als hoofd van het biochemisch laboratorium fungeert.

STELLINGEN

1 Bij het gebruik van preparaten, bestaande uit F(ab')2 fragmenten van antilichamen dient men het gehalte aan niet gesplitste antilichamen te bepalen en aan te tonen dat bij de gebruikte doseringen de toegediende hoeveelheid van deze antilichamen beneden de laagste nog actieve dosis ligt.

2 Tijdelijke toediening van cyclosporine A leidt bij de muis niet tot een definitief verlies van het vermogen tot afstoting van een huidallotransplantaat.

3 Met antisera gericht tegen producten van het H-2L locus kan acute afstoting worden geinduceerd.

4 De bij de muis van nature voorkomende proteinurie wordt niet veroorzaakt door een vergrote doorlaatbaarheid van de glomerulaire basaalmembraan.

5 Anti-thymocytenserum opgewekt in een konijn verdient in de klinische transplantatie de voorkeur boven een preparaat, opgewekt in een paard.

6 Bepaling van de hoeveelheid paraproteine in humaan serum volgens de methode beschreven door Salmon en Smith is alleen mogelijk indien dit paraproteine in een elektrisch veld een uitgesproken gamma-mobiliteit heeft.

Salmon, S.E., and B.A. Smith. (1970). J.Clin.Invest. 49:1114.

7 Voor een adequate diagnostiek van monoclonale gammopathieën is het wenselijk dat immunofluorescentie en immunoelektroforeseonderzoek naast elkaar in hetzelfde laboratorium plaatsvinden.

8 Het gehalte aan transferrine in liquor cerebrospinalis stijgt met de leeftijd.

9 Immunologie is in de scheikundestudie ondervertegenwoordigd. Het is wenselijk om het volgen van een bijvakstage in deze richting te stimuleren.

10 Nieuwbouw in kleine kernen hoeft er niet toe te leiden dat de verschillen in stedebouwkundige identiteit tussen de afzonderlijke kernen vervlakken.

11 Een onderwijsbeleid dat gericht is op gelijke kansen in het onderwijs is gedoemd te falen zolang de gelijke kansen in de maatschappij niet verwezenlijkt zijn.

J. van Kemenade (1974). Beleidsplan voor het onderwijs aan groepen in achterstandssituaties. Staatsuitgeverij, 's-Gravenhage.

12 Het beschikken over veel motivatie en minder intelligentie levert vaak meer op dan de combinatie weinig motivatie, hoge intelligentie.

20 december 1979 R.M.W. de Waal