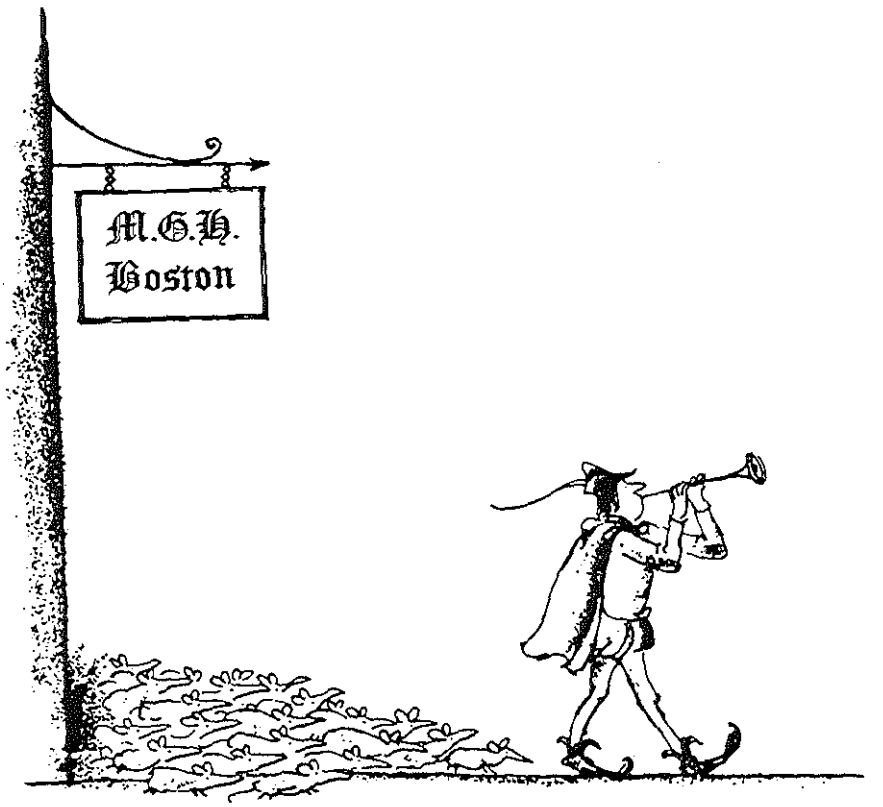
IMMUNOLOGICAL ENHANCEMENT OF SKIN ALLO- AND HETEROGRAFTS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IMMUNOLOGICAL ENHANCEMENT OF SKIN ALLO- AND HETEROGRAFTS

PROEFSCHRIFT

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CHAPTER I

INTRODUCTION

Since the development of transplantation of tissues, much effort has been directed towards the acceptance of transplanted grafts. Many methods have been developed to prevent or delay the rejection reaction. Some of them are used in clinical transplantation, involving immunosuppression of the host by certain drugs, hormones and by anti lymphocyte serum, which contains antibodies directed against the lymphocytes of the host. These methods, if carefully applied, may indeed prevent rejection of the grafts in a proportion of the patients. However, treatment with these drugs can be deleterious for the host, for suppression of the immune response increases the susceptibility for infection and probably also for tumors [10b]. In fact, many recipients of organ transplants still die from sepsis [261].

Thus, any type of immunosuppression is still associated with a noxious effect on the host. A solution for these transplantation problems might be provided by the oldest principle in the achievement of graft acceptance, namely enhancement. Recently Snell [257] defined *enhancement* as: the enhanced or prolonged growths of allografts, due to the presence in the graft recipient of allo-antibody directed against the allo-antigens of the donor [257]. In other words, a graft is protected by enhancing antibodies, that are not directed against the recipient as is the case for A.L.S., but against the donor antigens.

Evidence has been accumulated for a more biological function of immunological enhancement. For example, enhancement might be responsible for the occurrence of tumors in man [112], the favourable results in human kidney transplantation and for the acceptance of the foetus during pregnancy [114] as suggested by the findings in this thesis (chapter VIa). Even tolerance might in fact be mediated by enhancing antibodies [113].

Enhancing antibodies are, in contrast to the present immunosuppressive treatment, probably not harmful for the recipient, and thus might constitute a better solution for the problems in clinical transplantation. Enhanced tumor growth has always been the most reproducible example of the phenomenon. The efforts to induce immunological enhancement of grafts of normal tissue were until recently largely unsuccessful. In order to increase the possibilities of eventual clinical application of immunological enhancement, more information on the exact requirements to induce enhancement of a normal tissue graft is clearly needed. This is the very purpose of the experiments described in this thesis.

Skin was chosen as graft model, because skin grafting is simple to complete

and the outcome of enhancement of skin grafts seems a better indicator for the clinical situation than that of tumor grafts. In the first 4 experiments enhancement was studied with skin allografts, i.e. skin grafts exchanged between members of one species. Special mouse strains have been used in these four experiments in such a combination, that donor and recipient only differed at one single histocompatibility locus. Such a monospecific disparity was chosen on purpose, for enhancement might then be obtained more easily. Immunological enhancement of skin heterografts was tested in the rat species.

The reason that most of our experiments were done with mice as the laboratory animal of choice, is, that in this species the genetics of tissue transplantation have been studied most extensively. In addition, the largest number of inbred strains is available within this species, which enables extensive studies of transplantation immunology.

CHAPTER II

THE REJECTION REACTION

Immunological enhancement is supposed to have a certain effect on the immune reaction. Therefore, some aspects of the immune reaction will first be described, before discussing the problems around immunological enhancement.

Immune reactions are elicited by antigens, which are foreign to the host. Transplantation of normal or neoplastic tissue confront the genetically different recipient with foreign antigens and the ensuing immune response results in rejection of the transplanted tissue. These antigens, which are mainly located on the cell surface, are the so-called transplantation antigens. The severity of the rejection reaction depends upon the degree of histo-incompatibility, i.e. the disparity between donor and recipient, which is determined by multiple genes, the *histocompatibility genes* or H-genes. It has been shown for several species that the most important histocompatibility antigens are governed by genes of two closely linked loci. A graft will be permanently accepted when all histocompatibility antigens of donor and host are identical as is the case for identical twins and animals of inbred strains. The structure of the transplantation antigens is not yet completely understood. Its immunological specificity is under the control of genes at many loci, which are most extensively studied in mice. At least 15 H-loci are present in this species, of which one chromosomal region, the *H2-locus* is the strongest locus, as evidenced by the rate of rejection of skin grafts [258, 256] (table 1). Over 30 specificities have already been identified in this locus. The H2-antigens are present on virtually all cells, but especially concentrated on the lymphoid cells. Comparable loci in man, rats, Rhesus monkeys and dogs are respectively the *HL-A*, *Ag-B*, *RhL* [10b] and *DL-A* locus [286, 288b]. HL-A antigens however are not detectable on erythrocytes, while the erythrocytes of rodents do contain tissue antigens in sufficient quantity to make them demonstrable with special serological techniques.

The rejection of a graft involves a complex combination of *humoral* and *cellular* response [230, 231]. The cellular response seems to constitute the most important part of the acute rejection [233]. In 1944, Medawar described an infiltration of predominantly mononuclear cells during skin graft rejection [180]. Attempts to transfer immunity to skin grafts with serum failed consistently, whereas adoptive transfer of immunity appeared to be possible with sensitized lymphoid cells [27, 184, 185, 200]. Earlier, it was shown that the delayed hypersensitivity reaction, which is similarly caused by a cellular reaction [18, 236, 277a], can be adoptively transferred by mononuclear cells

Table 1. The H2-locus**

H2 alleles	Specificities (old symbols at top) A	D^b	C	D	E	F	G	H	I	J	K	M	N	P	Q	S	V	Y	A′	B′	C′	D′	E^d	D^k	K^b	inbred strains
$H2^a$	1	–	3	4	5	6	–	8	–	10	11	13	14	–	–	–	–	25	27	28	29	–	–	–	–	*A* AKR.K
$H2^b$	–	2	–	–	5	6	–	–	–	–	–	–	14	–	–	–	22	–	27	28	29	–	–	–	33	$C_{57}BL/_{10}$ $C_{57}BL/_6$ C_{57}L STA 129 C_3H.SW CC_{57}BR CC_{57}W D_1.LP
$H2^c$	*	–	3	4	5	*	–	8	–	*	–	13	*	–	–	–	*	*	27	38	29	–	*	*	*	D_1.C
$H2^d$	–	–	3	4	–	6	–	8	–	10	–	13	14	–	–	–	–	–	27	28	29	–	**31**	–	–	DBA/2 BALB/c C_{57}BL/Ks $B_{10}.D_2$ ST.T6 WH YBL/Rr YBR/Wi
$H2^e$	–	–	3	–	5	6	–	–	–	–	–	13	*	*	*	–	*	25	27	28	29	30	*	*	*	$STOL_1$
$H2^f$	+	–	–	–	–	?	7	8	9	*	–	–	*	–	–	–	*	–	27	–	–	–	*	*	*	A.CA B_{10}.M
$H2^g$	–	2	–	–	–	6	*	?	*	*	–	–	14	*	–	–	22	*	*	*	*	*	31	–	–	H2G
$H2^h$	1	2	3	–	5	6	*	8	*	*	11	–	?	*	–	–	–	*	*	*	*	*	–	–	–	H2H
$H2^i$	*	–	3	4	5	6	*	*	*	*	–	13	*	*	–	–	22	*	*	*	*	*	–	–	33	H2I
$H2^j$	–	–	–	–	–	6	?	–	*	–	–	–	–	*	–	–	22	?	?	*	*	*	*	*	*	JK/St
$H2^k$	1	–	3	–	5	–	–	8	–	–	11	–	–	–	–	–	–	25	–	–	–	–	–	32	–	AKR C_3H CBA CH_1 C_{57}BR/cd C_{58} D_1.ST MA MA/MY CE RF STB 101 C_{57}BR/a
$H2^l$	–	–	–	–	–	6	?	–	*	10	–	–	–	*	–	–	22	–	?	?	*	*	*	*	*	I/St N/St (?)
$H2^m$	*	–	3	–	5	–	–	8	–	*	11	13	*	–	–	–	*	*	27	28	29	30	*	*	*	AKR.M
$H2^n$	1	–	–	–	5	6	*	8	*	10	–	–	14	*	–	–	–	*	?	?	*	*	*	*	*	F/St
$H2^p$	*	–	3	–	5	6	–	–	–	*	–	–	*	16	–	–	*	*	–	–	–	–	*	*	*	P C_3H.NB BDP(?)
$H2^q$	–	–	3	–	5	6	–	–	–	–	11	13	–	–	17	–	–	*	27	28	29	30	*	*	*	$DBA/_1$ B_{10}.Y(?) C/St BUB
$H2^r$	*	–	3	–	5	–	–	8	–	*	11	–	*	–	–	–	*	25	–	–	–	–	*	*	*	RIII/Wy LP.RIII(?)
$H2^s$	*	–	3	–	5	6	7	–	–	*	–	–	*	–	–	19	*	–	–	28	–	–	–	–	–	A.SW SJL

* untested

** from "Biology of the Laboratory Mouse" [256]

[59, 158]. However, there is also evidence for rejection by antibodies only, as will be discussed later in this chapter.

In view of the purpose of this work, namely the investigation of the immuno inhibiting effect of enhancement, it seems useful to divide the immune reaction into three different components. Thus it will be possible to speculate where in the immune reaction the enhancing antibodies exert their inhibiting effect (fig. 1).

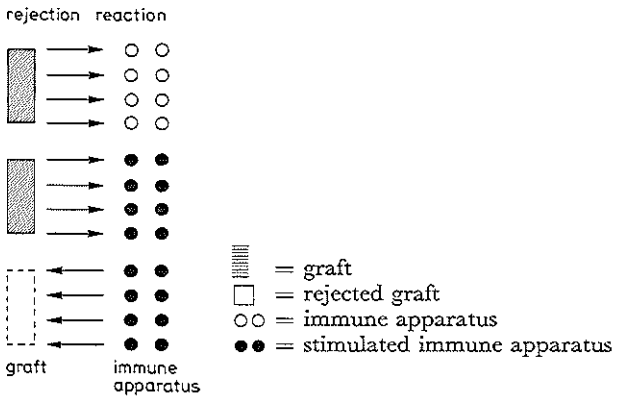


Fig. 1. Simplified scheme of rejection reaction

1. Afferent component: the transport of antigenic information to the immunocompetent cells.
2. Central component: the processing of the antigens and the activation of the lymphoid tissue.
3. Efferent component: the appearance of humoral and cellular factors in the circulation and their subsequent activities which lead to graft rejection.

1. Afferent component

It is not known how exactly the antigenic information reaches the immunocompetent cells. These cells may either move to the antigens or the antigens may be transported as free molecules or bound to recipient cells. Some evidence exists that the antigens are transported via the lymphatic drainage to the regional lymphe nodes. Recently Barker and Billingham provided more arguments in favor of this concept by showing that skin flaps, devoid of lymphe drainage, will not sensitize the host [11, 12].

Many investigators attribute the conveyance of antigens to the macrophages, that also seem to play a role in retaining certain antigens for longer periods of time [233]. Results of some in vitro [226a] and in vivo [82] studies

suggest that lymphoid cells need macrophages to elicit an immune response. Noltenius e.a. [208] even attribute an antibody producing quality to the macrophages. They found antibody producing macrophages with a modified Jerne-plaque technique (see page 63). In addition, an immunosuppressive effect of anti-macrophage serum has been demonstrated, but a significant efficacy could only be achieved following stimulation with low antigen doses [5, 76].

Antigens which stay in the lymphe nodes, remain either extracellular in the follicles or intracellular in the medullary macrophages [1]. The persistance of antigen is supposed to be required for the complete differentiation of immunocompetent cells into antibody forming cells [264, 209, 1]. Indeed some authors [102, 280] demonstrated that the presence of antigens is prerequisite for the continuous synthesis of 19S- and 7S-antibodies.

2. Central component

Although it is not yet known in detail how the antigens stimulate those lymphoid cells, which will initiate the rejection reaction, much information on this subject has been recently accumulated.

The antigen-sensitive cell. The lymphocytes which respond to antigenic stimulation are called the antigen sensitive cells or immuno-competent cells. The way they respond is represented in a xyz-scheme [45]. The antigen sensitive cell or x-cell becomes by antigenic stimulation a sensitized progenitor cell or y-cell. The y-cell is transformed into antibody producing z cells by further contact with the antigens [264]. Obviously this scheme does not represent more than a very crude representation of the totality of cellular changes that arise during antigenic stimulation. Some theories exist about the way the x–y-transformation of the antigen sensitive cell occurs.

Receptor site. It is suggested that the antigen is recognized by a special receptor site of the antigen sensitive cell [172, 218, 239, 295]. The structure and binding capacity of this receptor site is probably similar to the antibody which that cell will finally produce. A controversy exists about the amount of receptor sites present on one antigen sensitive cell. Some favor the concept of multipotential immunocompetent cells [214], others give credence to the existence of specific receptor sites on individual immunocompetent cells [207]. McConnell e.a. [214] specifically differentiated between IgM like receptors and IgG like receptors on rosette-forming cells in mice.

As early as 1935 the lymphocytes were recognized as the source of antibody production. The indirect fluorescence technique revealed that plasma cells contain antibodies. Some investigators now claim that both lymphocytes as well as plasma cells can produce antibodies. Lymphocytes probably transform

into plasma cells [104]. Singhal [245] suggested that the immune response is initiated in the bone marrow, but completed in lymph nodes and spleens.

Much of the initial information about the immunocompetent cell came from studies of the *Graft Versus Host* reaction. A Graft Versus Host reaction occurs when lymphoid cells are injected into allogeneic or heterogeneic recipients which are unable to reject those lymphoid cells. The grafted cells react immunologically to their new host. In this way a reversed homograft reaction occurs, that is characterized by a "wasting disease". Small lymphocytes were shown to convey the Graft Versus Host activity [93, 94, 122]. Hence they are thought to represent the immunocompetent cells.

In fact the small lymphocytes appear to be precursors for the cells that regulate antibody production and are also responsible for the so-called cell-mediated graft destruction [56, 173]. Gowans [93] isolated small lymphocytes from the thoracic duct and demonstrated that these cells continuously recirculate between lymphe-channels and the blood stream. This cell population has a very extended life span and carries the immunologic memory [92].

The stem cells for the immunocompetent cells are situated in the bone marrow. Following antigenic stimulation, many small lymphocytes transform into large lymphoblasts. The thymus has a regulatory role. Namely the cell-mediated reaction is, in contrast to the humoral response, predominantly thymus dependent, and is depressed by neonatal thymectomy. Thus the cellular response is mediated by Thymus-dependent lymphocytes (T-cells) [198]. These *T-cells* are small lymphocytes that recirculate in the blood stream and are concentrated in the paracortical areas of the lymphenodes. Conversely the humoral response is bone marrow dependent. The bone marrow dependent areas are located in the cortical zone of the lymphenodes, where the *B-cells* can be detected, or plasma cells that are responsible for the humoral response.

3. Efferent component

During the efferent phase, the actual rejection takes place by a combination of humoral and cellular reactions [186]. It is not yet known how the activated small lymphocytes or T-cells kill the cells of the graft [226b, 277a], but one assumption is, that these cells secrete noxious factors following contact with target cells. Such an intensive cell to cell contact has been reported [197]. There is also evidence for interaction between lymphocytes and macrophages by which phagocytosis occurs. Sensitized lymphocytes probably produce a factor, the Migration Inhibiting Factor (M.I.F.), that inhibits the migration of macrophages.

The role of the humoral reaction in the rejection of skin and tumor grafts is thought to be of minor importance, but will be more extensively discussed in view of the characteristics of enhancing antibodies. Participation of immuno-

globulins in the rejection reaction has been described. Firstly, neoplastic cells of reticular tissue are *in vivo* as well as *in vitro* more sensitive to the cytotoxic action of antibodies, than other tumor cells. Resistance to humoral antibodies seems to be related to the concentration of isoantigen on cell surfaces [187, 189]. Cells more densely covered by antigenic sites are more vulnerable.

Secondly, several investigators described a deleterious effect on grafts by passive transfer of immunity by antiserum [15, 61, 63, 64, 91, 106, 107, 204, 263, 265, 266, 267, 284]. Hasek [106] reported the destruction by antiserum of a skin graft present on a duck tolerant to the heterologous donor duck. Recently, the abolition of tolerance by antiserum was also demonstrated in rats [109, 166]. Najarian and Feldmann [204] and Kretschmer and Perez-Tamayo [156, 157] showed accelerated rejection of skin grafts in mice and rats respectively by immune lymphoid cells kept in a diffusion chamber. Yet the antibodies alone could never account for the graft rejection observed in those experiments, where the recipients still have an intact immune apparatus, for then the cellular response may play a major role in rejection. Evidence for a synergistic effect of immune lymphoid cells and antibodies has been provided by Batchelor [15]. A clear cut effect of serum alone was obtained in the rejection of kidney allografts in dogs by Clark e.a. [63]. They transferred the kidney, after implantation into specifically sensitized irradiated dogs, back to the original donor. Rejection occurred then within two hours. Also in clinical transplantation, hyperacute reactions are observed if the recipients possess antibodies directed against the donor antigens.

A dual effect of serum has also been shown [44, 88]. The growth of a mouse Ehrlich ascites tumor in guinea pigs was inhibited when incubated with specific xenogenic γ_2-immunoglobulin and enhanced with the $F(ab)_2$-fragment of the serum.

Thus, by the injection of antiserum, either an accelerated rejection or enhancing effect can be expected. For clinical transplantation it would be of great value to be able to eliminate those factors of the serum that lead to rejection. Therefore the enhancing properties of antisera were tested in the experiments that are described in this thesis.

Suppression of the rejection reaction

Whereas the rejection reaction is still not completely understood, many studies have already been undertaken to find methods for the prevention of the occurrence of this reaction. Clinical transplantation would indeed not be possible without the use of immunosuppressive agents, like corticosteroids, Azathiopurine and Actinomycin. In recent years, an effective serum has been developed by injecting lymphoid cells of the recipient into allogeneic or heterogeneic animals. This anti lymphocyte serum (A.L.S.) is currently being

tested in clinical trials. Although these methods are to some extent effective in preventing a rejection reaction, one principal drawback of their use is the noxious effect on the recipient.

A safer way to achieve immunological unresponsiveness might proceed from two immunological phenomena, which have been known for a long time, namely immunological enhancement and tolerance [160, 179, 182]. The following definition of *tolerance* has been given by Medawar: "If an animal is exposed to an antigen before it has developed the capacity to react against it, the development of that capacity is delayed and, under certain circumstances, may be indefinitely postponed." Immunological enhancement, as mentioned before, is mediated by specific antibodies directed against the donor antigens. Clinical application is not yet feasible, because of the limited knowledge about these phenomena, that even might be closely related [113, 163]. In the presented experiments immunological enhancement of normal tissues was studied. The results may lead to a better understanding and, eventually, to clinical application of this interesting modification of the immune response.

CHAPTER III

IMMUNOLOGICAL ENHANCEMENT

1. History

Enhancement of tumors

Enhancement has been described by Kaliss [139], one of the early investigators of this phenomenon, as "the successful establishment of a tumor homograft and its progressive growth (usually to death of the host) as a consequence of the tumor's contact with specific antiserum in the host". As with so many new discoveries this was detected by chance in the course of investigations into the immune therapy of cancer. In the beginning of this century it was already known, that after the rejection of a transplantable tumor, immunity had been developed against a second inoculation of the same tumor [86, 90]. Attempts to produce immunity by injections of non living tumor tissue, succeeded in some instances. Frequently however, a paradoxal effect was noted. The expected accelerated rejection of the test graft did not occur after pretreatment with either frozen, lyophilized or otherwise killed tumor tissue of the same antigenic structure.

The first to describe this phenomenon were Flexner and Jobling in 1907 [86], who reported the progressive growth of a rat sarcoma in some rats, which had previously rejected a first graft of the same tumor. Later, enhancement was studied mostly with transplantable tumors indigenous to inbred strains of mice or rats. One of the first systematic investigations with non-inbred animals was undertaken by Casey in 1932 [49, 50, 51, 52, 53). Using the transplantable Brown-Pearce rabbit epithelioma, he induced its progressive growth in allogeneic hosts by previous injections of frozen tumor tissue. This *xyz*-effect, as he called it, was tumor-specific because inoculations of frozen Brown-Pearce tumor did not affect the growth of a mouse carcinoma or sarcoma. Surprisingly the "enhancing material" was still effective when injected two weeks after the tumor inoculation. Rabbits injected 8–10 times with this substance even exhibited enhancement of a subsequent tumorgraft 7 months later! The author did not try to exclude tolerance as the cause of his findings, for tolerance was not yet known at this time.

Following Casey's work, which already demonstrated so many characteristics of the enhancement phenomenon, numerous investigators have explored this field.

The characteristics of the tumor

The importance of the characteristics of the tumor grafts was pointed out by

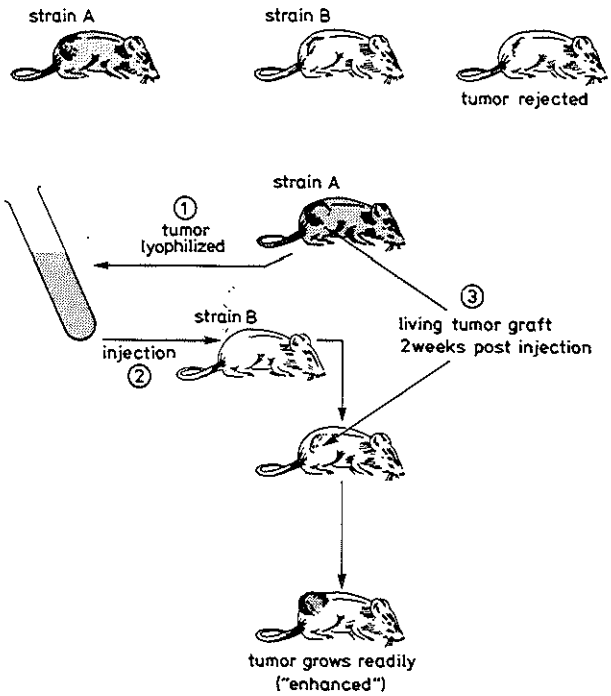


Fig. 2. Active enhancement of tumor grafts

Snell e.a. [252]. Pretreatment with specific lyophilized tumor tissue in mice, enhanced the growth of strain A carcinoma in 100% of the treated animals (fig. 2), whereas a leukemia was rejected by the same specific enhancing treatment. A series of injections of lyophilized tissue prior to the isogenic tumor inoculation gave various results amongst 9 different tumors, studied in 7 different strains of mice [253]. Again growth of the leukemia was inhibited. Later it was shown that enhancement of leukemia did not occur because of the exceptionally high sensitivity of cells of lympho-reticular origin to the cytotoxic action of antibodies.

With other tumors enhancement occurred to various degrees, depending on host strain and tumor type. The growth of fibrosarcoma L 946 was either inhibited or stimulated, depending on the strain of mice in which it was inoculated. Of two strain A tumors, Sarcoma I and the 15091[a] tumor, a more consistent and significant enhancement was obtained with the Sarcoma I [139]. For this reason this Sa I tumor was often used in the classical experiments of Kaliss [132, 140]. Inoculation of the Sa I tumor of A mice in C_{57}BL/Ks mice first led to a period of heightened resistance starting on the third day and subsiding after 2–3 weeks. Only afterwards enhancement of a second graft was observed.

According to the theory of Möller [190], the different behaviour of the various tumors is determined by the concentration of antigenic receptors on the surface of the tumor cells. This hypothesis will be discussed later in more detail.

The condition of the tumor-cell

Inoculations of killed tissue as initial immune stimulus more often led to enhancement than when a live graft was used. The enhancing substance was present in the frozen tumors used in Casey's experiments and in the lyophilized tissue of Snell [252, 253, 254] and Kaliss [144]. The enhancing factor could not be isolated by differential centrifugation nor by tumor filtration through Selas or Berke-field filters [254] or by ultrafiltration of tumor supernatant [135].

Theories

The variety of experimental models and of subsequent results did not lead to a better understanding of this phenomenon but instead brought about considerable confusion. Casey related the "xyz"-effect to a growth stimulating factor, present in tumors. Kaliss [134, 143] thought of "conditioning the host", and Billingham, Brent and Medawar [23] of "actively acquired tolerance".

At this point a phenomenon, probably related to enhancement, namely *antibody mediated humoral unresponsiveness*, should briefly be discussed. This phenomenon comprises the suppression of the humoral response towards various antigens by antibodies directed against those antigens. Many authors have postulated a similarity between this phenomenon and the process of immunological enhancement. However, it should be kept in mind that the antibody mediated humoral unresponsiveness is essentially concerned with humoral immunity, while the rejection of grafts, – in particular those of skin and tumor tissue – is primarily effected by a cellular immune response. Suppression of the latter is therefore equally important from the clinical point of view.

Yet, the data on antibody mediated humoral unresponsiveness cannot be disregarded entirely in the discussion on clinical transplantation because the suppression of both the humoral- and the cellular response by antibodies could be based on the same principle. Therefore some examples of antibody mediated humoral unresponsiveness will be presented throughout this chapter, although it is not implied that we are necessarily dealing with an identical mechanism. A more detailed discussion will follow at the end of this chapter.

Specific immunological effect

It was the merit of Kaliss and co-workers to clarify to some extent the mystery

concerning the phenomenon of enhancement. First, they showed that pretreatment with normal homologous tissue could also lead to enhancement of subsequently inoculated tumor tissue of the same strain [142]. Furthermore, they demonstrated that the effect of the pretreatment was dose-dependent. In a certain mouse strain combination, where the graft normally grows in 50% of the untreated recipients, "conditioning of the host" could lead to immunity when very small doses of antigen were used, while larger doses caused enhancement [133, 134]. These observations did not favour the idea of a growth stimulating substance being the active principle, but rather suggested a specific effect of the petreatment on the immune mechanism. Although Casey was the originator of the "growth stimulating substance" hypothesis, his own results in fact supported this latter view, which will be discussed below.

Enhancing antibodies

Conclusive evidence for the involvement of the immune system was provided by Kaliss: Antiserum prepared in $C_{57}BL/Ks$ mice by 6 intraperitoneal injections of Sarcoma I tumor enhanced its growth, when injected seven days prior to the inoculation of the tumor in an untreated C_{57}/Ks mouse [132] (see fig. 3).

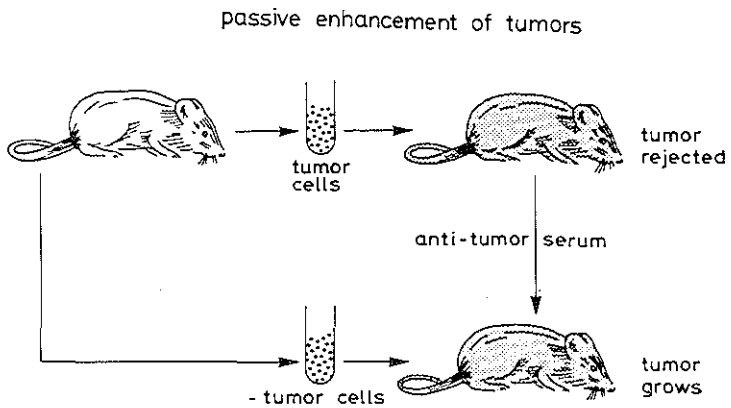


Fig. 3. Enhancement of tumor grafts by passive transfer of anti-tumor serum

The passive transfer of enhancement, or passive enhancement is since a crucial test in enhancement studies (fig. 3).

In certain circumstances pretreatment with tumor or normal tissue apparently has such an effect on the immune system, that antibodies are formed with enhancing capacities. These antisera specifically prolong the survival of those grafts, that are isogenic with the very antigens that elicited the antibodies [136, 137]. The activity of these sera is associated with the γ-globulin fractions [138, 141, 145].

Still, the questions remained, how these immunoglobulins are produced, how they exhibit their protective action and which antibodies or fragments are involved. The protection of a graft by antibodies seems to run counter to the evidence of their participation in the rejection reaction [15, 63, 107, 204].

The occurrence of accelerated rejection of a tumor, injected within the first two weeks after the inoculation of a live allogenic tumor graft, plus an increasing number of surviving tumors, when grafting was performed later, suggested that enhancing antibodies occur rather late after immunization [57, 140]. Enhancement of tumor grafts has even been reported after an interval of 40 weeks after the first inoculation [50]. These findings suggest that more than two weeks after tumor inoculation, antibodies with enhancing capacities are formed. The production of these enhancing antibodies may be continued for 40 weeks. For comparison the peak of hemagglutinin production was found to be at about 15 days after the inoculation of the tumor and a titer was present for at least 8 months [138].

Dosage and antibody titer of serum

The minimum *dosage* of antiserum effective in passive enhancement is often quite small. 0.1 µl of antiserum daily was sufficient in several experiments [14, 67, 89, 110, 126]. Some evidence even exists that small amounts of antiserum give a better passive enhancement [88]. Kaliss [139] noted a suppression of antiserum in volumes of 0.2 ml and an enhancement of growth with smaller amounts. Furthermore, Möller [190] reported either an inhibiting or enhancing effect by the use of large doses and varying degrees of enhancement with small doses. Only one injection of antiserum is often sufficient. It can in certain cases successfully be injected as long as 10 days after tumor grafting [140].

A correlation between the *antibody titer* against donor cells and the effectiveness of antiserum has been observed by some authors. Moore and Pereira [199] noted that after active immunization of mice, the occurrence of lower levels of antibodies correlated with immunity and higher levels with enhancement. Other investigators [14, 57, 155] also described very high antibody levels in concurrence with active enhancement in mice. A similar trend has been reported with passive enhancement, namely that hyperimmune sera with high haemagglutinating titers were most effective [2, 271].

Antibody-structure [168]

There is still no general agreement about which type of antibody and which fragments are responsible for the enhancing phenomenon.

The antisera are usually obtained from hyperimmunized animals. The

enhancing quality of antibodies mostly increases with time after immunization and with the number of immunizations [67, 71]. In most animal species the first antibodies to appear after a primary antigenic stimulus are the 19S-globulins (*IgM*), followed by the 7S-immunoglobulins (*IgG*) [85, 153, 280]. Repeated immunizations lead to the formation of IgG [153]. Hence one would expect those golbulins to be responsible for enhancement. Indeed the 7S-immunoglobulins were found to be responsible for the induction of tumor enhancement [28, 272, 273, 276] and the inhibition of the immune response of antigen stimulated animals [195], whereas the 19S-antibodies were 100–200× less effective [193]. IgG is also more effective in antibody mediated humoral unresponsiveness. The 7S-globulins of a homologous hyperimmune antiserum to bacteriophage $\vartheta\chi 174$ in guinea pigs were much more effective that 19S-antibodies to prevent antibody formation to χ bacteriophage [84].

The 7S-immunoglobulins can be separated in a fast and slow portion, respectively the IgG-γ_1- and IgG-γ_2-fraction. Tokuda and Mc Entee [276] and Irvin e.a. [129] more specifically denoted the slow IgG-fraction, the 7Sγ_2-globulin as the agent responsible for passive enhancement of Sarcoma tumor in mice. This was confirmed by Takasugi and Hildemann [272, 273]. In their experimental model, which will be further discussed later on, 7S-γ_2 was responsible for enhanced tumor growth in mice. IgG-γ_1 and IgM were not effective in this respect. Haemagglutinating properties were found in IgM and IgG-γ_1 and IgG-γ_2. Only IgM and IgG-γ_2 had cytotoxic properties. It had already been shown by Nussenzweig [210] that IgG-γ_2 was able to cause haemolysis and he concluded that only IgG-γ_2 can bind complement. In contrast to these findings Voisin [284] previously had reported the enhancing effect of the fast 7S-γ_1-fraction of mouse sera. Again cytotoxicity was associated with the slow fraction.

Fab- and Fc-fragments

Fab- and Fc-fragments can be obtained by digesting γ-immunoglobulins with proteolytic enzymes like papain. The antigen binding capacity is located on the Fab-fragment. The Fc-fragment contains the structures responsible for complement fixation. The Fab- and Fc-fragments of the γ-globulins have been investigated with respect to their enhancing effect.

Chard e.a. [58] induced enhancement of EL_4-leukemia in mice by Fab-preparations, which also inhibited the cytotoxic effect of antibodies in vitro. These investigators used a cell type, which is unique in enhancement experiments, since cells of normal or neoplastic lymphoid tissues are highly sensitive to cytotoxic antibodies. Furthermore, Broder and Whitehouse [41] showed that the growth of a mouse Ehrlich ascites tumor in guinea pigs was inhibited, when incubated with specific heterologous γ_2-immunoglobulins and enhanced

with the $F(ab)_2$-fragment of the same serum. The Fc-fragment of an otherwise enhancing serum was also ineffective in the experiments of Tao and Uhr [274]. The effectiveness of $F(ab)_2$-fragment in the enhancement of renal allografts in rats has recently been demonstrated [240a]. Hence the Fab-fragments seem to be responsible for the enhancing effect, which might be related to the fact that they contain the combining sites for antigen.

According to these findings the effect might take place at the peripheral level by binding to the antigens and not through a direct effect on the antigen-sensitive cells. By binding to the antigen the Fab-fragments may mask its antigenic character.

In contrast with such reasoning are the results of Sinclair e.a. [243], who demonstrated that $F(ab)_2$-antibody alone was much less effective than whole antibody in inhibiting the primary hemolysin response to sheep erythrocytes. Other investigators [96, 129, 272, 273] found evidence for an effect of the Fc-fragment in enhancement, which is a rather unexpected finding, for the Fc-fragment represents the receptor site for complement fixation and thus is essential for a cytotoxic effect. All these contradictory results obviously did not lead to a better understanding of the mechanism of immunological enhancement.

2. Mechanism

The paradoxical influence of antiserum, namely the protective effect of certain humoral antibodies, instead of their participation in rejection, is still not well understood.

Several hypotheses are proposed for the mechanism by which pre-existing or passively transferred antibody suppresses the immediate and/or delayed hypersensitivity. The early theories stem from the time that enhancement was only elicited with tumors.

a. Physiological alteration of the graft

One of Kaliss' first hypotheses involved a non-immunological change of the tumor graft, that allows it to survive despite a hostile response of the host [140, 3].

b. Immunoselection

By immunoselection those cells that are more compatible with the host are allowed to survive, whereas the other cells are killed by specific antibodies.

These two theories are now mostly abandoned. The hypotheses which are currently under investigation, will be discussed below.

c. Inhibition of the rejection reaction

Immune inhibition can take place at the following stages (fig. 4):

1. The afferent arch, i.e. the process of recognition of the graft-antigens by host immunocytes.
2. The central component, which involves the stimulation of the antigen-sensitive cells, through which an immune response arises.
3. The efferent part, in which the graft is subjected to the attack of the cellular and humoral reactivity of the host.

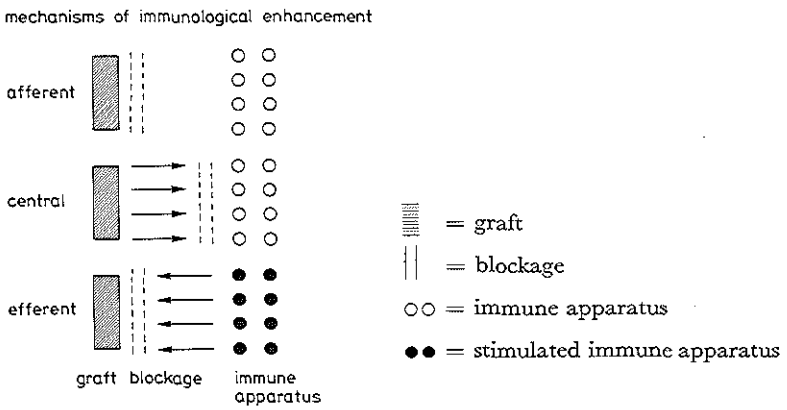


Fig. 4. Simplified scheme of possible mechanisms of immunological enhancement

It should be taken into account, that the experiments, which are cited, are all of a different design. Therefore opposite results do not necessarily contradict each other.

Some of the findings in the literature, that contribute to the solution of the enigma of enhancement, will be summarized in four tables (tables 2, 3, 4, 5). However our interpretation of the results as presented in these tables is not always the same as that given by the various authors.

1. Afferent inhibition (see table 2: afferent/central inhibition)

Following this theory, the host is unable to recognize the allograft as foreign, because of a masking effect by the specific antibodies. The antigens of the graft are either inactivated, or their release is prevented. Thus an immune response will not be elicited in the host, as is also the case with central blockage of the immune response. Therefore, a differentiation between afferent and central inhibition could not be made in the various experiments (table 2).

The absence or depression of the immune reaction during enhancement has been described by several authors. Snell [251] and Mc Kenzie [175] reported a depression of immune reactivity of the draining lymphe nodes. Tagasuki [272, 273] noted the absence of lymphocytosis in enhanced mice. In addition, a delay in humoral response has also been observed [126, 189, 194, 251]. The

Table 2. Evidence for an afferent and/or central blockage of the immune response by enhancing antibodies

				Afferent - Central	
authors	year	model	graft	findings	mechanism
Snell	'56	mouse allografts	tumor	rejection instead of enhancement by addition of donor lymphocytes to the tumor graft	afferent + central + efferent −
Mitchison	'55 '56	mouse allografts	tumor	abolishment of enhancement by adoptive transfer of immunity	afferent + central +
Möller	'63				efferent −
Billingham	'56				
Hutchin	'67	mouse allografts	tumor	slower rise of haemagglutinating titers during enhancement	afferent + central +
Möller	'63				
Möller	'64				efferent −
Snell	'60				
Snell	'60	mouse allografts	tumor	depression of cellular immunity of the draining lymphe nodes by the addition of anti donor serum to the tumor cell inoculum	afferent + central + efferent −
Takasugi	'69	mouse allografts	tumor	no lymphocytosis during enhancement	afferent + central + efferent −
Bloom	'70				
McKenzie	'70	mouse allografts	skin	draining lymphe nodes from enhanced mice show less reactivity	afferent + central + efferent −

absence of an adequate cellular response seems to be responsible for the occurrence of enhancement in these experiments. Consequently enhancement might be abolished by restoring a normal immune response. Indeed, enhancement of tumor grafts in mice did not occur, when the tumor cells were mixed with lymphocytes from the same donor strain [250]. Apparently the antigenic strength of the lymphocytes had broken through the masking effect of the enhancing antibodies. Enhancement of tumor grafts could also be abolished by adoptive transfer of immunity to the enhanced mice [22, 133, 142, 184, 188]. This excludes the presence of an efferent blockage.

Some evidence for the existence of an afferent inhibition was provided by Möller [190]: Normal rejection by A.CA mice of a tumor of the $(A \times A.CA)F_1$ strain did not occur after in vitro incubation of tumor cells with 0.1 ml A.CA anti-A serum. In the enhanced mice the antibody response was delayed for 5 days. Apparently, "coating" of the tumor cells with antibodies induced enhancement and delayed the humoral response. This suggests an inhibition at the afferent level. Yet an efferent blockage could not be excluded, as an immune reaction did finally occur.

In contradiction to a purely afferent blockage of the immune response is the immunosuppressive effect of antibody injections 6–8 days (71, 272, 273] or 10 days [140] after the administration of the antigen. Such a time interval is – according to several authors [84, 164, 181] – sufficient to initiate an immune response. In other words, at that time the immune response is already elicited and the antibodies can only counteract its effect at the central or efferent level.

2. Central blockage of the immune response (table 3)

Central blockage may involve the blockage of either the processing of antigens by macrophages, the transmittance of antigenic information to the antigen sentitive cell, or the transformation of the immunocompetent cells (x-cells) into sensitized y-cells and antibody producing z-cells. Following this theory, inhibition of the host's immune mechanism occurs at the central level by antibodies, which are raised in the strain of the host and directed against the donor. This suggests a feed-back mechanism. The immune response is blocked by the very antibodies that were produced during this response.

Table 3. Evidence for a central blockage of the immune response by enhancing antibodies

					Central
authors	year	model	graft	findings	mechanism
Batchelor	'62	mouse allografts	tumor	very small amounts of antiserum	afferent —
Gorer	'61			can produce enhancement	central* ±
Haughton	'69				efferent —
Hutchin	'67				
Amos	'70	mouse allografts	tumor	immunological reactivity of immune lymphoid cells was lost after contact with antibody coated tumor cells	afferent — central + efferent —

* ±: no definite proof

In most experiments the existence of a pure central blockage could not be adequately shown. Whereas a central inhibition could not be excluded in the experiments favouring a blockage at the afferent level, a differentiation between an efferent and central mechanism was often not possible either (table 4). In vitro and in vivo experiments, in which the inhibition of tumor growth by immune lymphoid cells could be prevented by antibodies, were suggestive of a blockage at the central or efferent level [2, 14, 126, 298, 299]. Recently Amos [2] introduced a variant of this so-called Winn-assay. He showed that mouse lymphoid cells, after incubation with enhancing antibodies, maintained a normal function. However, when instead of antibodies, antibody-coated tumor cells were incubated with the lymphoid cells, no immunologic activity was left in the lymphoid cells after separation from the coated tumor

Table 4. Evidence for a central and efferent blockage of the immune response by enhancing antibodies

					Central-Efferent
authors	year	model	graft	findings	mechanism
Takasugi	'69	mouse allografts	tumor	Antidonor serum still active when	afferent −
Kaliss	'58			injected 6–10 days after grafting.	central +
					efferent +
Amos	'70	mouse allografts	tumor	Not any inhibition of tumor	afferent −
Batchelor	'62			growth by immune lymphoid	central* ±
Hutchin	'67			cells, when anti-donor serum is	efferent +
Möller	'63			added to a mixture of graft cells and immune lymphoid cells.	
Hellström	'70	human tumor-bearers	melanoma	Prevention of destruction of tumor cells by immune lymphoid cells in vitro, by addition of antiserum of tumor bearers.	afferent − central* ± efferent +
Möller	'63	mouse allografts	tumor	Enhancement of tumor cells, coated with antibodies, in immunized recipients.	afferent − central* ± efferent +

* ±: no definite proof

cells. An injection of incubated lymphoid cells together with fresh tumor cells even resulted in enhancement. The author proposed that an immunosuppressive substance (I.S.S.) may be released from either the antibody-coated tumor cells, or the lymphoid cells after their exposure to the antibody-coated cells. Yet antibodies might have attached to the lymphoid cells during the incubation with the antibody-coated tumor cells.

Takasugi and Hildemann [272, 273] offered some interesting data, which suggests an impaired immunity during enhancement in mice. The Sarcoma I tumor from the A strain was tested in A.BY hosts. Hyperimmune A.BY anti Sa I serum induced progressive growth of the tumor. The lymphocytosis occurring during the normal rejection of the tumor grafts was absent in mice displaying enhanced tumor growth, suggesting a central or afferent inhibition of the immune reaction. Yet it is questionable whether a depression at the central level reveals itself specifically by the abscence of lymphocytosis. Suppression of lymphocytosis could still be achieved by administration of enhancing antibodies on the 6th day after transplantation but not by surgical removal of the tumor at that time. The authors therefore strongly favoured the occurrence of a central blockage. However, the tumor cells could already have dispersed into the circulation or local lymphe nodes during the 6 days after inoculation. In such a way sensitization of the host might have continued after removal of the tumor. Thus an afferent inhibition in the enhanced mice could not convincingly be excluded.

An argument against a peripheral effect is the observation that very small

amounts of antibody up to 0.0005 ml, can produce enhancement [14, 67, 89, 110, 126]. Similarly, the protection against Graft Versus Host (G.V.H.) disease by passively transferred anti-host antibodies, can not be easily explained by "coating" of all the cells of the host. The abscence of a G.V.H. reaction by passive "facilitation immunologique" was noted by Voisin [282] and confirmed by others [275]. Haughton [110] calculated that the number of antibody molecules necessary to cover the antigenic sites of 5×10^8 sheep red blood cells (S.R.B.C.), was $100\times$ higher than those needed for suppression of the immune response. Furthermore, total antibody coating of tumors may be rapidly reduced by tumor cell division and Amos [2] showed that the antibodies, attached to the tumor cell surface, will be detached within 6 hours at body temperature.

These observations oppose an afferent and efferent, or respectively masking and "walling off" effect, and are in disagreement with the theories of Möller, who favoured a pure peripheral effect. This author [187, 188, 190] studied the different sensitivities to antibodies of various tumors as mentioned earlier in this chapter. He observed that large doses of antibody caused enhancement of some tumors and rejection of others, whereas small doses of antibody induced enhancement to various degrees. He related this phenomenon to the amount of antigenic receptors on the surface of the tumor cells. Sensitive tumors have many surface antigens and will be easily damaged by high antibody and complement concentrations, in contrast to the resistant tumor with few antigenic receptors. Thus a quantitative rather than a qualitative difference seems to exist between sensitive and resistant cells. This concept seems to be discordant with the finding that it is relatively difficult to enhance leukemia's, which consist of cells known to be highly sensitive to cytotoxic antibodies. If enhancement depended on the appropriate coating of tumor cells, enhancement of leukemia's should also be possible. Interestingly enhancement of leukemia's has indeed been reported in the last decade [31, 58, 188].

Suppression of the cellular response has been noted with antigens other than those provided by skin, tumor- or vascularized-grafts.

The first report about inhibition of delayed hypersensitivity came from Crowle and Hu [67]. Antiserum from guinea pigs or mice, hypersensitized to chicken ovalbumin, suppressed the immediate and delayed hypersensitivity reactions to the ovalbumin in mice. Rowley e.a. [228], studying antibody mediated unresponsiveness with sheep red blood cells, also claimed to have some evidence for the depression of the cellular response by antiserum [227]. In contrast, several investigators demonstrated a normal capacity of antiserum treated lymphoid cells to produce antibodies, when transferred to irradiated or normal isologous mice [181, 191, 233, 292].

Evidence that enhancing antibodies can have some biological impact on lymphoid cells was provided by Greenberg and Uhr [96]. Passively transferred

Table 5. Evidence for an efferent blockage of the immune response by enhancing antibodies

authors	year	model	graft	findings	Efferent mechanism
Cepellini	'70	human allografts	skin	Enhancement of grafts by in vitro	afferent —
Möller	'64	mouse allografts	tumor	incubation with antiserum.	central —
Halasz	'65	rabbit allografts	skin	Normal rejection of non-incubated graft in the same recipient.	efferent +
Hellström	'71	human tumor-bearers		In vitro abolishment of cell mediated immunity of the patient by incubation of tumor cells and the serum of the patient.	afferent — central — efferent +
Chantler	'67	mouse allograft	tumor	Adoptive transfer of immunity by cells from mice exhibiting enhancement.	afferent — central — efferent +
Berne	'65	mouse allograft	tumor	Enhancement of tumor graft with	afferent —
Haskova	'62		skin	normal rejection of skin graft.	central — efferent* ±
Morris	'70	rat allografts	kidney	Lymphocytes from enhanced rat responded normally in M.L.C..	afferent ± central — efferent +

* ±: no definite proof

antibodies stimulated the protein synthesis in lymphoid organs of immunized rats, measured by 14C-leucine incorporation. A correlation however between this effect and immunosuppression could not be shown.

3. *Efferent blockage* (table 5)

Following this theory, the graft antigens are not destroyed in spite of a normal immune reaction. The cell surface is coated by specific antibodies, in such a way that a protective effect at the target-cell level is exerted. Möller [188, 187] arrived at this conclusion by the finding that enhancement of tumors could be passively induced in previously sensitized animals. Yet a central inhibition was not excluded in these studies.

Conclusive evidence for the existence of a block at the efferent level was provided by several authors [55, 99, 196, 112, 115], by *in vitro incubation* of the graft with antibodies. They demonstrated in different experimental models the presence of a normally functioning immune apparatus during enhancement. Grafts, that were incubated in vitro with specific antibodies enjoyed a prolonged survival time in untreated recipients, whereas a simultaneously transplanted non-incubated graft from the same donor was normally rejected. Möller [106] showed this with tumor grafts in mice. Cepellini [55] obtained in one case prolongation of human skin graft survival in an A.B.O. incompatible but H.L.A. identical situation, by in vitro incubation of a skin graft with

antiserum, whereas the simultaneously grafted control skin graft from the same donor was normally rejected. Halasz [99] perfused one ear of a rabbit with homologous antiserum. Skin grafts from the perfused ear and the other ear of the same rabbit were simultaneously transplanted to another rabbit. Skin grafts from the perfused ear survived significantly longer than those from the non-perfused ear.

Hellström e.a. [112, 115] noted in man the destructive effect of lymphocytes from tumor bearing donors on plated tumor cells in an in vitro colony inhibition test. Incubation of the tumor cells with serum of the patient abolished the cell mediated immunity of the lymphocytes in vitro. The factors in the serum that are responsible for this blocking effect may very well be 7S-immunoglobulins and operate at the efferent level. Thus all these experiments provide strong evidence for protection by specific antibodies of tumor- and skin grafts in mice, rabbits and man.

An efferent blockage was less convincingly demonstrated in a few other experiments. Lymphoid cells from enhanced animals were shown to be immunologically active: adoptive transfer of immunity by cells from mice exhibiting enhancement was demonstrated by Chantler [57]. Lymphocytes from enhanced rats responded normally in the Mixed Lymphocyte Culture (M.L.C.) [201]. A normal immune response during enhancement has also been suggested for mice that displayed enhancement of tumors concomitantly with a normal rejection of skin grafts [108, 21]. However, different grafts were compared in these experiments.

In conclusion, a general agreement has not been reached about the mechanism of enhancement, in the sense that it has been difficult to demonstrate a blockage of the immune response at one single level. Most convincing in this respect were the findings of Möller [196], Halasz [99] and Cepellini [55], which seem to provide direct evidence for an *efferent* inhibition.

3. Enhancement of normal tissues

Skin grafts

Tumors were mostly used for the study of enhancement. A striking difference exists in the tendency of skin and of tumor grafts to undergo enhancement. Several investigators attempted to obtain prolongation of skin allograft survival by active or passive enhancement, but only a few were slightly successful.

Active enhancement of mouse skin allografts has been reported by Billingham e.a. [22]. Pretreated A mice tolerated a CBA skin graft 4.8. days longer than the controls. Brent and Medawar [32] noticed a slight increase in skin graft survival by passive transfer of serum across the H2-locus in mice, judged by six-day epithelial survival scores.

Möller [191] reported *passive enhancement* of a mouse skin graft of 1.5 days, when donor and recipient differed at H2- plus non H2-loci, and 2.8 days, when the histo-incompatibility consisted of the H2-locus only. A similarly slight passive enhancement of mouse skin grafts was obtained by Kodama e.a. [155] and Chutna [60]. Passive enhancement of skin grafts has also been obtained in other species, like rats [65, 119, 301, 302], guinea pigs [206], rabbits [99, 260] and man [55]. A significant prolongation of about four days was reported by Heslop [119] in rats. However, only a weak histocompatibility difference existed in this host-donor combination. Significant results were obtained by Zimmerman and Feldman [301, 302] with a pronounced prolongation of rat skin allograft survival in more than 50% of neonatal recipients. One must realise though, that immature rats were used in these experiments, which facilitated an inhibition of the immune reaction [120]. Also in non inbred guinea pigs [206] and rabbits [99, 260] a slight passive enhancement of skin allografts could be obtained. Finally, Cepellini [55] described one case of enhancement of human skin grafts as mentioned previously (p. 00). By passive immunization in an HLA-identical but ABO incompatible combination, a skin graft survived 9 days longer than a control graft.

An explanation has not been found yet for the resistance of skin grafts to enhancement. For a strong enhancement, the antibodies should be directed against all the antigens of the graft [71, 189, 192]. The antisera, used in the experiments with skin grafts, in general raised by injections of spleen cells and/or lymphocytes, did not meet these requirements, for tissue specific antigens probably do exist (30, 111, 242). Most experiments on enhancement of tumor tissue on the other hand, were indeed performed with anti-tumor sera.

If this conception is valid, enhancing sera should be raised by immunization with the specific graft-tissue antigens although lymphocytes are present in the epidermis [4]. In this respect, the experiments of Benkö e.a. [19] should be mentioned. They produced a rabbit anti-rat skin serum, which as they claimed, did contain antibody against a skin specific antigen, which was not present in the rat plasma. This serum induced an unresponsiveness towards allogenic rat skin grafts. Similarly Nelken and Cohen [205] reported prolonged survival of rat skin allografts after incubation with rabbit and chicken anti-rat skin sera. However, it should be born in mind, that such sera can also be regarded as unconventional antilymphocyte sera and the observed effect could be due to that property.

Renal allografts [202, 203, 220]

Much better results have been obtained with the enhancement of renal allografts (table 6). One of the first reports came from Halasz [101]. *Dog* kidney allografts survived significantly longer after pretreatment of the recipient with

Table 6. Enhancement of renal allografts

author	year	active/ passive	en- hance- ment	model	M.S.T. controls	M.S.T. experimental group
Halasz	'64	active	±	canine allografts	8.1	29
Wilson	'69	active	±	canine allografts	9	144
Wilson	'70	active	±	canine allografts	9.4	44
Stuart	'68	active+ passive	+	rat–AgB locus		>100
French	'69	passive	+	rat	8.5	>120
Morris	'70	passive	+	rat–AgB locus		~
Ockner	'70	active	±	rat–AgB locus		>120
Zimmerman	'70	active	±	rat–AgB locus		~
Marquet	'70	active	±	rat–AgB locus	12	100
Batchelor	'70	passive	±	human–Bγ26 locus		> 90

±: no definite proof for enhancement

subcutaneous injections of 2 ml donor blood. A differentiation between enhancement or tolerance as the underlying mechanism was not attempted. Later donor blood has been successfully used as pretreatment for *porcine* liver allografts [46] and renal allografts in *rats* [167]. Marquet [176] obtained prolongation of renal graft survivals in rats up to 350 days and permanent acceptance of heart allografts.

Prolonged canine renal allograft survival by pretreatment with donor spleen cells has also been reported by Wilson e.a. [296, 297].

Recently very extended survivals were obtained across the major AgB-locus in rats, by pretreatment with either bone marrow cells [211, 212] or spleen cell fractions [301, 302]. Definite proof for enhancement has however not been provided in either of these studies on active enhancement. It remains possible that tolerance is the cause of the extension of graft survival in these experiments.

The first to describe the *passive* transfer of enhancement by serum with renal allografts was Stuart [269, 270]. He obtained a long survival of rat kidney allografts by injections with donor antigens, anti-donor serum, or both, across the strong AgB-locus in rats. In the same experimental model indefinite survival was recently described by Morris e.a. [201]. French and Batchelor [87] induced indefinite survival of (August × AS) kidney allografts in AS rats by passive transfer of anti-donor serum. Antibody titers were shown in the rats displaying enhancement. Apparently, sensitization of the recipients had also occurred.

Batchelor [17] even applied the principle of passive enhancement to a *human*

kidney transplantation. The antiserum was raised in the father of the patient by injections with cells of the donor "casu quo" the mother. Fab-fragments of this serum were injected into the recipient, who received a kidney from the mother, which only involved one HLA-incompatibility. The kidney functioned well, but a difficulty in the interpretation of his results arises from the use of immunosuppressive drugs postoperatively.

Interestingly, in a few cases a rat skin graft transplanted to a recipient tolerating a kidney graft from the same donor strain, was rejected within normal time [201, 211, 212]. Yet, in the case of enhancement, one would expect either acceptance of a second graft or a concomitant rejection of skin and kidney, unless enhancement is mediated either by protection of the antigens at the efferent level or by organ specific antibodies. It might also be possible that enhancement is more easily induced with vascular grafts.

Such a difference in rejection patterns between skin and kidney grafts in rats has been reported previously in cases with weaker genetic disparities. White and Hildemann [289, 290, 291] showed this in the Fischer-Lewis combination. Without any treatment, kidneys of the Fischer strain were accepted by Lewis rats for as long as 32 weeks, whereas Fischer skin grafts were rejected in a median survival time of 10–13 days. This phenomenon, as Sakai [235] pointed out, occurs in many weak rat strain combinations. Self enhancement as proposed by White and Hildemann, is not an obvious explanation, for enhancing antibodies are specifically directed against all the donor antigens and hence also against the skin. Adaption or a lesser vulnerability of the kidney grafts fits in better with the different findings in these experiments. A stronger sensitization with skin grafts than with vascularized organs may well play a role. This difference is due to the fact that lymphatic vessels are more important for the sensitization process than blood vascular channels [12]. Similar results have been reported for heart [11] and liver grafts [46].

Thus one should not rely too much on the results obtained with vascular grafts across weak histocompatibility barriers as these results can be due to several other mechanisms rather than enhancement. In particular they should not lead to the conclusion that clinical application of enhancement is justified at this time.

4. Antibody-mediated humoral unresponsiveness

The reason for discussing antibody mediated humoral unresponsiveness in this same chapter, is its possible relation with enhancement. It comprises the depressing effect of passively administered specific antibodies on the humoral response to simultaneously injected antigens. In the beginning of this century, studies directed at the prevention of infectious diseases revealed that a mixture of toxoid and excess antibodies did not result in active immunity [248]. It was

not until 50 years later that this phenomenon was studied more extensively with various antigens e.g. viruses, bacteria, red cells, toxins and proteins. Uhr and Möller wrote an excellent review about this subject [281].

The suppression of the humoral response was either assessed by the level of antibody titers in the serum or the number of antibody producing cells in the spleen. The two tests give information about the humoral immunity and will be discussed concurrently. The *plaque forming cell technique* of Jerne and Nordin [131] enables the enumeration of antibody producing cells, which form hemolytic plaques in a layer of sheep red blood cells (S.R.B.C.). These plaque forming cells (*P.F.C.*) appear after a latent period of 24 hours and reach their maximum 4–5 days after immunization [47]. Using this test, the effect of anti-S.R.B.C. serum in mice [117, 196, 200, 193, 192, 293] and rats [227, 228] was thoroughly studied. Much of the information about antibody mediated humoral unresponsiveness arose from these studies. It was established by many investigators that the suppressive effect of antibodies is specific for the antigens concerned [20, 42, 188, 229, 278, 291, 291].

One of the first investigations about the paradoxical depression of humoral reaction by its own products, came from Uhr and Baumann [278, 279] in their experiments. Excess antibody was favourable for the occurrence of suppression of the humoral antibody response. Injections of diphtheria toxoid together with antitoxin excess in rabbits, rats or guinea pigs did not provoke the expected primary antitoxin response. The authors suggested a feed-back mechanism effectuated by the binding of antibodies to the antigens, which in such way became inactivated.

Another hypothesis has been proposed by Hanna et al [103] i.e.: antibodies block the conversion of antigen sensitive x-cells to sensitized y-cells and to antibody producing z-cells, by covering of the antigens. By this blockage a maturation arrest occurs in the xyz-transformation. This induces an expansion of the y-cell compartment, that is responsible for the secondary response [100, 102]. This theory is based on the assumption that antibody formation and immunological memory are dependent upon the continuous presence of antigens [280, 293, 294].

The question arises whether this feed-back mechanism also occurs in a more physiological situation. Morris and Möller [200] showed that not only artificially administered antibodies, but also endogenously formed antibodies can induce humoral unresponsiveness. In their experimental model immune or hyperimmune mouse spleen cells adoptively transferred to syngeneic recipients, suppressed the 19S-synthesis to S.R.B.C. as measured by P.F.C. The decrease in IgM-formation by 7S-antibodies has also been demonstrated by some other investigators [193, 234, 280]. It appears that a prevention of excess antibody formation is thus part of a more general homeostatic mechanism.

Initially it seemed that only IgG was responsible for this phenomenon [84,

67], but various investigators have subsequently demonstrated the effectiveness of IgM [193, 227, 293].

IgG not only seems to be more effective in the induction of humoral unresponsiveness, but moreover sensitized 7S forming cells are more resistant to inhibition [84, 193, 234]. The development of P.F.C. is most easily inhibited by antibodies administered before or during the induction of antibody synthesis and is not affected during a second set response. Yet, antibody mediated suppression of the 7S-response is possible. Even long after the peak of IgG-production, its inhibition could be induced by special batches of IgG, obtained late after immunization [294]. 7S-antibodies seem to be more effective in inhibiting antibody formation after an increasing number of immunizations [71, 84, 287], indicating their qualitative change with time [294].

These findings suggest a relation between the *affinity* of antibodies for antigenic determinants, – which occurs late in immunization – [79, 246] and the capacity to inhibit the humoral immune response. This was confirmed by Walker and Siskind [287], who noted a much better suppression of high affinity antibodies than of low affinity antibodies. The antibodies however have to compete with preformed antibody present on the surface of certain antigen sensitive cells [74, 295]. The existence of the antibody-like receptors for antigens on lymphoid cells was demonstrated by the selective binding of immune cells in glass bead columns, which were coated with specific antigens [295].

The antigen combining site of these cell-associated antibodies seems to be similar to that of the antibodies which will finally be produced by those cells after immunization. An increase in affinity for antigens with time also occurs with these cell-associated antigen-specific receptors. Thus only high affinity antibodies can succesfully compete with high affinity cell-associated antigen-specific receptors and this circle of events may well constitute the feed-back mechanism under discussion. Consequently, the suppression of a secondary immune response by antibodies will be much more difficult, because of the high avidity of the competing receptor sites of the sensitized cells for antigen [200, 227, 277, 292].

Another explanation for the mechanism of humoral unresponsiveness is put forward by some investigators, who suggest a direct effect of antibodies on the immune competent cells [83, 96, 227, 228, 244]. In addition to humoral unresponsiveness, suppression of the cellular response is also reported [8, 67, 68]. Delayed type hypersensitivity to S.R.B.C. in rats was partially abolished by either active or passive immunization, but completely by a combination of antigen and antibody injections [7, 8]. The inhibition of a cellular response is of course of importance for eventual clinical application of this phenomenon.

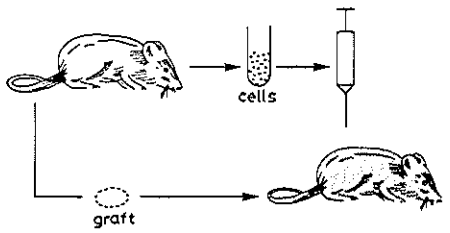
In conclusion, the bulk of the experimental data suggests that an important step in the suppression of the humoral response is the interaction of antigens

and antibody. This conclusion is substantiated by the specificity of the unresponsiveness and the effectiveness of antibodies with high affinity for antigenic determinants. Apparently, high affinity antibodies preferentially bind with antigen, in competition with those immunocompetent cells that contain receptors on the cell surface, which have a structure similar to that of the antibodies. The significance of this feed-back mechanism for the organism as a whole is still not well understood, but it seems clear that antibodies play an important role in the regulation of the antibody formation. A similar mechanism may be effective in the prevention of Rh immunity by means of anti-Rh antibodies [62].

PART II
EXPERIMENTS AND DISCUSSION

CHAPTER IV

ACTIVE ENHANCEMENT OF SKIN ALLOGRAFTS IN MICE



1. Introduction

Enhancement is, as Kaliss showed [132], mediated by specific antibodies, directed against the graft and has been thought to be completely different from *tolerance* [160, 178, 182]. Efforts to obtain enhancement of skin grafts were only slightly successful [22, 34, 60, 119]. On the other hand much of the work on tolerance has been successfully performed with skin grafts. Pretreatment of adult recipients with a variety of donor tissues resulted in specific graft acceptance, which was mostly ascribed to tolerance [72, 178]. Either homogenates, cell fractions, extracts or viable cells were used [60, 169, 170, 178] via intraperitoneal (i.p.) or intravenous (i.v.) injections [22, 120, 240b]. Prolonged survival of skin allografts in adult mice has been reported across the strong H2-locus by injections of high doses of donor-spleen cells [22, 169, 240b, 34]. Halasz e.a. [99] demonstrated prolonged survival of kidney-allografts in dogs by subcutaneous (s.c.) injections of donor blood prior to transplantation.

This immunological unresponsiveness is mostly thought to be induced by tolerance [178]. However, partial and total immunological unresponsiveness towards a graft after pretratment with donor antigens could be caused by either *active* enhancement or tolerance. Indeed a few authors reported passive transfer of the immunological unresponsiveness by serum which strongly suggests that enhancement is responsible for the effect [60, 20].

The purpose of the current experiments was to achieve more pronounced enhancement of skin grafts in mice in view of the mediocre results obtained previously with enhancement of skin grafts. Skin grafting is an easy and fast procedure and should thus be an advantageous model for the study of enhancement.

To facilitate the attainment of enhancement a model was designed in which the host differed from the donor at one single histocompatibility antigen of the strong H2-locus. This difference was strong enough to induce a fast rejection in

untreated hosts. B_6AF_1 (31^-) mice ($C_{57}BL/_{6J} \times A/JaxF_1$-hybrids) were injected with viable or frozen-thawed $B_{10}D_2$ (31^+) cells as a pretreatment for a $B_{10}.D_2$ skin graft. In this combination a monospecific histo-incompatibility exists, constituting a so-called *co-isogenic* combination. The $B_{10}.D_2$ mice are isogenic with the B_6AF mice except for the *31*-specificity of the H2-locus (table 1).

Two major subdivisions of the H2-region can be discerned in mice. These are the "*D end*" and the "*K end*" respectively [275b]. The 31-specificity is following Snell e.a. [258b] a "private" H2-specificity, located at the K end of the H2-chromosomal region.

2. Material and methods

Animals

B_6AF_1 (31^-) mice were recipients of cells or skin from $B_{10}.D_2$/new Sn (31^+) female mice. This is a coisogenic combination. Before applying a $B_{10}.D_2$ female skin graft, frozen-thawed or viable female $B_{10}.D_2$ tissue was injected s.c., i.p or i.v. into B_6AF_1 mice. The B_6AF_1 mice were divided into different groups. Each member of a group was treated with cells from the same suspension. All the mice in these and the following experiments were more than seven weeks old and purchased from the Jackson Laboratory, Bar Harbor, Maine. Females and males were used separately.

Preparation of the cell suspension

Viable cells. For the preparation of viable cells, spleens and lymphe nodes were collected in medium 199 and then gently pressed through a stainless steel mesh. Cell counts were performed in a haemocytometer and viability determined by exclusion of Trypan blue.

Non-viable suspensions. Spleens and lymphe nodes in most cases mixed with livers, were collected in medium 199, pressed through a stainless steel mesh and frozen in dry ice with alcohol.

After freezing and thawing three times, viable cells could not be detected. After spinning down, a 20% solution in saline was made by mixing in a blender.

To prevent intravascular clotting, 125 U.S.P. units sodium heparin were added to 1 ml of the suspension. The 20% solution was injected once a week, either slowly intravenously into the tail vein or s.c. or i.p. Of the frozen-thawed suspension 0.25 ml was used per injection unless otherwise stated. The test-skin grafts were applied one day after the last injection.

Skin grafting

Skin grafts were performed following the technique of Billingham and Medawar [24]. The graft bed was prepared with curved scissors, leaving the panni-

culus carnosus muscle and the vascular fascial planes immediately overlying the panniculus intact. Grafts of 1.5×1.5 cm were placed on the back of the recipient, then covered with vaseline petrolatum gauze and held in place by plaster bandage. Plasters were removed on the 8th day and the grafts inspected every other day by macroscopical examination. Rejection was characterized by complete destruction of epithelial surface.

Antiserum

Serum used for passive transfer was collected from B_6AF_1 male mice, which had been treated with three i.v. injections of frozen-thawed $B_{10}.D_2$ tissue. These mice were bled one day after the last injection from the sinus cavernosus of the eye. The blood was kept at room temperature until clotting occurred and afterwards at $+4\,°C$ overnight for further contraction of the clot.

Statistics

Statistical significance of the results was tested with methods of Student Welch and Wilcoxon. M.S.T. denotes Median Survival Time and $\bar{x}$ stands for the Mean Survival Time. The two tests had to be used together, for the distribution of the data was not always suited for the application of one single test.

3. Results

Controls

$B_{10}.D_2$ female skin grafts were rejected in 8 to 12 days (M.S.T. 9 days: s.d. 1.32) by 45 B_6AF_1 females. However, 24 B_6AF_1 males rejected the $B_{10}.D_2$ skin grafts later (M.S.T. 12 days; s.d. 1.21). Thus the difference in immune response between males and females manifested itself in these experiments in a different rejection period of skin grafts.

Subsequently the effect of pretreatment with injections of $B_{10}.D_2$ cells on the survival of $B_{10}.D_2$ skin grafts was studied. The donor cells consisted of either frozen-thawed or viable cells.

I. FROZEN-THAWED $B_{10}.D_2$ TISSUE

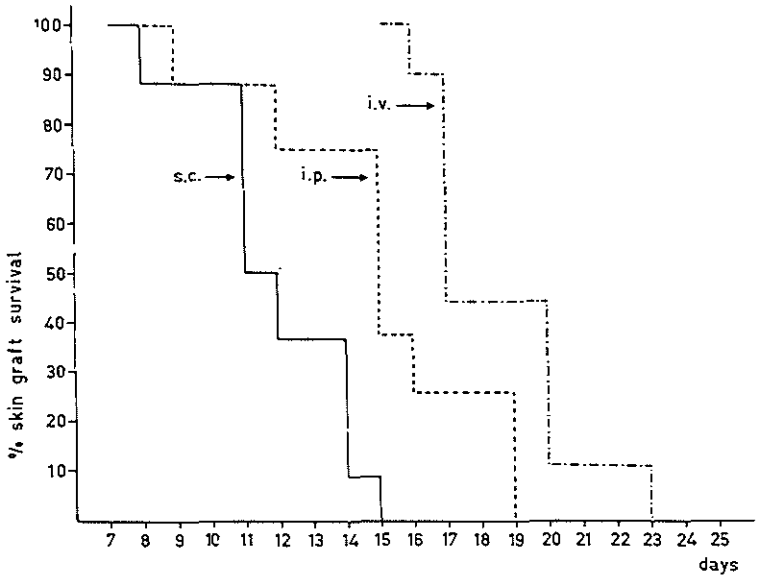
The results of injections with spleen, lymphe node and liver cells are described under *A*. The experimental groups resorting to *B* only received injections of cells from spleen and lymphe nodes.

A. SPLEEN, LYMPHE NODES AND LIVER

The bulk of the suspension used in these experiments was formed by liver cells.

Route of injection

Four subcutaneous, intraperitoneal or intravenous injections of the same cell suspension in 30 B_6AF_1 mice, resulted in a prolongation of the survival time of a subsequent $B_{10}.D_2$ skin graft of respectively 2.5, 6 and 8 days (fig. 5). Thus



Injections i.v., i.p. or s.c. Skin grafted 1 day after the last injection.

	injections	n	M.S.T.	s.d.	P_{Student}	P_{Wilcoxon}
	controls	45	9	1.32		
———	s.c.	8	11.5	2.49	< 0.02	< 0.09
– – – – –	i.p.	8	15	3.74	< 0.005	< 0.0002
—·—·—	i.v.	9	17	2.23	< 0.0005	< 0.0001

Fig. 5. $B_{12}.D_2$ skin grafts transplanted to B_6AF_1♀ after pretreatment with 4 injections of frozen-thawed $B_{10}.D_2$ liver cells, spleen cells and lymphocytes at weekly intervals.

no sensitization was obtained with these injections. The prolongation of graft survival was most strongly manifested with i.v. injections and least obviously with s.c. injection. Consequently only i.p. and i.v. injections were used in the experiments.

Number of i.p. and i.v. injections in B_6AF_1 females (table 7)

a. Intraperitoneal injections

The $B_{10}.D_2$ skin grafts did not survive for a longer time after pretreatment with 1, 4 or 5 i.p. injections. Only one group, which received 3 i.p. injections with Freund's adjuvant added, rejected a $B_{10}.D_2$ skin significantly later.

b. Intravenous injections

One or two injections did not lead to an extension of graft survival. Three

Table 7. Female B_6AF_1 mice transplanted with ♀ $B_{10}\cdot D_2$ grafts. Pretreatment with a different number of injections at weekly intervals of frozen-thawed ♀ $B_{10}\cdot D_2$ liver cells, spleen cells and lymphocytes, following various routes.

route	number of injections	n	M.S.T.	s.d.	$P_{Student}$	$P_{Wilcoxon}$
	0	45	9	1.32		
i.p.	1	7	9	1.51	0.80–0.40	< 0.4
	3*	9	18	3.08	< 0.0005	< 0.000003
	4	8	9	0.99	0.50–0.25	< 0.9
	5	5	8	1.52	0.30–0.15	
i.v.	1	10	9	0.98	0.50–0.25	
	1**	5	8	1.73	0.60–0.30	
	2	7	9	1.62	1.0	
	3	7	18	59.14		
	3	9	12	1.98	< 0.005	< 0.006
	3	9	12	2.38	< 0.005	< 0.005
	4	10	12	1.95	< 0.005	< 0.008
	5	10	14	5.16	< 0.01	< 0.00004
i.p.+i.v.	3+2	5	14	2.68	0.05–0.025	< 0.01
	3+3	5	10	3.67	0.20–0.10	< 0.04
	3+4	5	11	2.55	0.10–0.05	< 0.05
	5+2	5	10	2.83	0.40–0.20	

* plus Freund's adjuvant
** 0.05 ml/injection

injections however resulted in a significant prolongation of a subsequent skin graft. Two of the B_6AF_1 mice, that received three injections even did not reject the $B_{10}.D_2$ skin before day 135, beyond which time the grafts were not observed. Four or five injections were not more effective than three injections.

c. Intraperitoneal and intravenous injections

A combination of i.p. and i.v. injections was also effective but not as much as i.v. injections alone.

In conclusion, i.v. injections only lead to a consistent prolongation of graft survival. A correlation seems to exist between the number of injections and the occurrence of decreased immunological responsiveness.

i.v. injections in B_6AF_1 males

A more significant prolongation was expected to occur with the use of B_6AF_1 males as recipients, because of their weaker immune response, which is supposed to be easier overcome. This did not happen and the results were comparable to those in the females (table 8). Pretreatment with 2–6 i.v. injections resulted in a longer survival of subsequent skin grafts.

Time response

As mentioned before, all mice were grafted one day after the last injection. Of interest is the follow-up of the immune response of the immunized mice during

Table 8. Male B_6AF_1 mice transplanted with $B_{10} \cdot D_2$ skin grafts. Pretreatment with different numbers of i.v. injections of frozen-thawed ♀ $B_{10} \cdot D_2$ liver cells, spleen cells and lymphocytes.

number of injections	n	M.S.T.	$\bar{x}$	s.d.	P Student	P Wilcoxon
0	24	12	11.6	1.21		
2	10	14	13.7	2.50	< 0.02	< 0.005
3	10	15.5	18.1	6.12	< 0.01	< 0.0004
3	9	16	15.0	2.18	< 0.001	< 0.002
3*	10	12.5	12.2	1.75	< 0.30	< 0.5
5	10	17	16.3	2.94	< 0.0005	< 0.0002
6	10	13.5	14.2	1.75	< 0.0005	< 0.002

* plus Freund's adjuvant

Table 9. B_6AF_1 transplanted with ♀ $B_{10} \cdot D_2$ skin grafts at varying times after the last i.v. injection with frozen-thawed $B_{10} \cdot D_2$ liver cells, spleen cells and lymphocytes. Two groups: female and male B_6AF_1 recipients.

sex	number of injections	interval in days*	n	M.S.T.	$\bar{x}$	s.d.	P Student	P Wilcoxon
	0	0	45	9	9.4	1.32		
	3	1	6	11	11.3	1.97	< 0.05	< 0.06
	1 3	4	6	15	15.0	3.29	< 0.01	< 0.0002
	3	11	5	13	14.0	3.46	< 0.05	< 0.0004
	3	21	5	14	13.8	1.30	< 0.001	< 0.0003
♀	3	33	5	10	10.8	1.92	< 0.20	< 0.4
	3	1	5	14	16.0	5.20	< 0.05	< 0.0002
	3	5	4	10.5	10.75	0.96	< 0.10	
	2 3	9	5	10	11.0	2.0	< 0.20	< 0.25
	3	15	5	9	9.4	1.14	< 0.9	
	3	25	5	14	14.0	3.39	< 0.05	< 0.001
	0	0	24	12	11.6	1.21		
	3	1	7	14	14	3.41	< 0.10	< 0.02
	1 3	4	10	16	17.6	5.95	< 0.01	$\rightarrow 0.0003$
	3	22	9	14	14.1	2.26	< 0.01	< 0.008
♂	3	33	11	14	13.7	2.00	< 0.005	< 0.008
	4	1	10	11.5	11.9	2.51	< 0.70	< 0.6
	2 4	4	9	15	15.2	4.32	< 0.025	< 0.04
	4	6	6	13	13.12	2.14	< 0.20	< 0.2

* between last injection and skin grafting

a certain time after the last injection. This could give some information, as to whether enhancement or tolerance is the cause of this phenomenon [134].

a. Female B_6AF_1 mice

Two groups were treated with 3 i.v. injections and subsequently grafted at

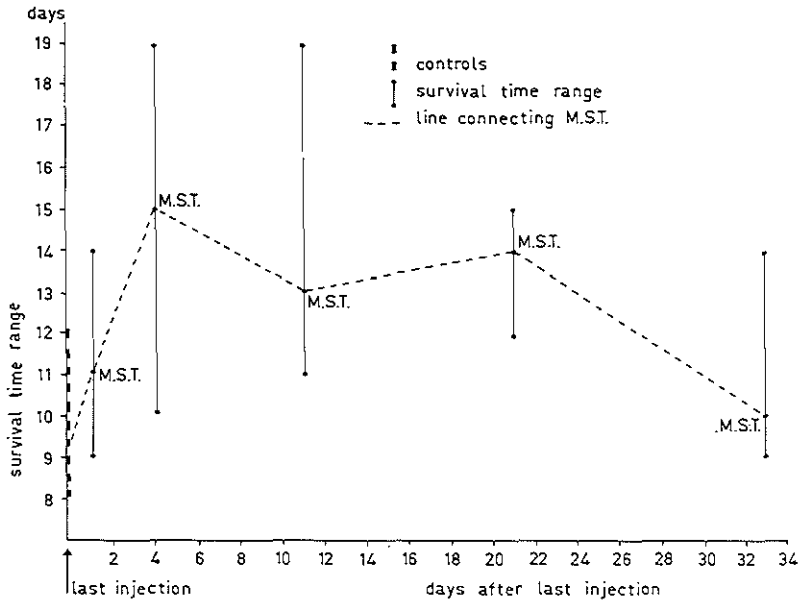


Fig. 6. $B_{10} \cdot D_2$ skin grafts transplanted to B_6AF_1 females at varying days after the third i.v. injection with frozen-thawed $B_{10} \cdot D_2$ liver, spleen and lymphocytes.

different times after the last injection (table 9). In the first group (table 9 and fig. 6) the prolongation of graft survival was most significant on the 4th day after the last injection and not on the 1st day. Grafting on the 21st day after the third inoculation resulted in a decrease in extension. The second group (table 9) displayed a different rejection pattern. Prolongation occurred after the first day with a $\bar{x}$ of 16 days (s.d. 5.20) followed however by a rapid decrease in prolongation from the 5th to 15th day. Afterwards an increase in survival time occurred.

b. B_6AF_1 males

With two groups of B_6AF_1 males the strongest prolongation of graft survival did not occur at the 1st day, but at or after the 4th day (table 9). This does not suggest tolerance as the cause, for in that case an immunological responsiveness was to be expected from the first day on. Yet a maximal humoral response could be expected from the 4th day on. Thus enhancement might be the cause of the prolongation of graft survival as immunological enhancement depends on the occurrence of antibodies.

Second set response

In this experimental group $B_{10}.D_2$ females were grafted without any pretreatment and subsequently were injected with frozen thawed tissue after the rejection of the first graft had taken place. A second set response is characterized

Table 10. Survival times of second ♀ $B_{10} \cdot D_2$ skin graft on B_6AF_1 female mice after pretreatment with i.v. and i.p. injections of frozen-thawed $B_{10} \cdot D_2$ liver cells, spleen cells and lymphocytes.

pretreatment	n	M.S.T.	$\bar{x}$	s.d.
0	45	9	9.4	1.32
1 skin graft	24	7	7	1.12
1 skin graft 4 i.p. injections	8	8.5	8.5	0.53
1 skin graft 4 i.p. injections 4 i.v. injections	5	13	60.4	69.05
4 i.v. injections 1 skin graft	5	7	9.2	3.03

by an early and strong reaction, which is not easily overcome by the various immunosuppressive drugs. In these experiments a second set response could be abolished by i.p. and i.v. injections of frozen-thawed tissue (table 10). A surprising effect appeared in a group of B_6AF_1 mice, which was treated after the first skin graft with 4 i.p. and 4 i.v. injections $B_{10}.D_2$ tissue. In this group two of the second $B_{10}.D_2$ skin grafts were not rejected at the 136th day.

B. SPLEEN AND LYMPHE NODES (table 11)

1%, 10% or 20% solutions of frozen-thawed $B_{10}.D_2$ tissue of lymphe nodes and spleens did not produce a significant prolongation of skin graft survival when injected in B_6AF_1 male mice. A slight sensitization was induced by i.p. injections. Thus the addition of liver tissue proved to be important for the achievement of immunological unresponsiveness, as injections with cells of spleens and lymphe nodes alone were not effective in these experiments.

Table 11. Male B_6AF_1 mice transplanted with female $B_{10} \cdot D_2$ skin grafts, 3 days after pretreatment with various solutions of frozen-thawed $B_{10} \cdot D_2$ spleen cells and lymphocytes.

route	solution	n	M.S.T.	$\bar{x}$	s.d.	P_{Student}	P_{Wilcoxon}
–	–	24	12	11.6	1.21		
i.v.	1%	10	12	12.3	1.77	< 0.30	
i.v.	10%	10	11	11.3	1.16	< 0.50***	
i.v.	20%	8	13	13.5	2.27	< 0.05	< 0.17
i.v.*	20%	10	11	11.4	1.50	< 0.80***	
i.v.	20%	10	12	12.0	2.79	< 0.70	< 0.8
i.p.	20%	10	10	10.3	1.42	< 0.025***	
s.c.	20%	10	10.5	10.7	1.64	< 0.20***	
i.p.**	20%	5	10	10.2	1.30	< 0.10***	

* skin grafted 1 day after last injection
** plus Freund's adjuvant
*** shorter survival time than controls

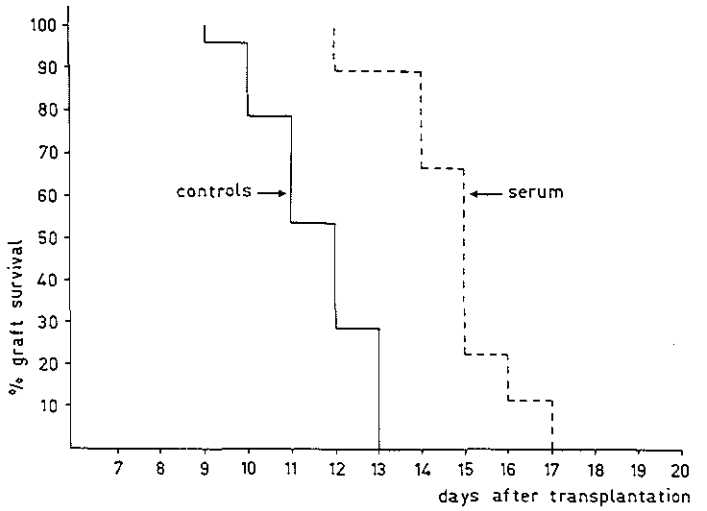
II. VIABLE $B_{10}.D_2$ TISSUE (table 12)

Pretreatment with *viable* $B_{10}.D_2$ lymphe node and spleen cells also resulted in an inhibition of the rejection reaction when more than two injections were used. One injection with 1, 18 or 30×10^6 viable donor cells however resulted in an accelerated rejection. In contrast to the previous experiments with frozen-thawed tissue, acceptance of skin grafts was in this experiment achieved without the addition of liver cells to the suspension.

Table 12. Female B_6AF_1 mice transplanted with female $B_{10} \cdot D_2$ skin grafts. Pretreatment with i.v. injections of *viable* ♀ $B_{10} \cdot D_2$ spleen cells and lymphocytes.

number of injections	dose	*n*	M.S.T.	s.d.	*P* Student Welch	*P* Wilcoxon
0	0	45	9	1.32		
1	1×10^6	4	8	0.57	< 0.0005 *	
1	18×10^6	4	8.5	0.57	< 0.02 *	
1	30×10^6	4	8.5	0.57	< 0.02 *	
2	18×10^6 40×10^6	8	10.5	2.20	< 0.30	
3	16×10^6 20×10^6 20×10^6	9	14	3.24	< 0.005	< 0.00008

* shorter survival



	n	$\bar{x}$	s.d.	*P* Student	*P* Wilcoxon
——— controls	24	11.6	1.21		
– – – – serum treated	9	14.8	1.39	< 0.0005	< 0.0001

Fig. 7. $B_{10} \cdot D_2$ skin grafts transplanted to B_6AF_1 males. Effect of the *passive transfer* of anti-$B_{10} \cdot D_2$ serum from actively enhanced B_6AF_1 mice.

Passive transfer of immunological unresponsiveness (fig. 7)

The question arises whether the effect of the injections with cells from the donor strain was related to enhancement or to tolerance. A crucial test for enhancement is its passive transfer by antibodies. Serum was collected from B_6AF_1 male mice, which were bled one day after the third injection with frozen-thawed $B_{10}.D_2$ tissue. Then 0.2 ml of this serum was injected every other day in B_6AF_1 mice until the day of rejection. $B_{10}.D_2$ skin grafts in these B_6AF_1 mice enjoyed a significantly prolonged survival up to 17 days. The prolongation was comparable to those obtained by injections with donor celles in the previous experiments.

4. Discussion

In the present experiments the effect of administration of donor cells prior to skin grafting was studied.

A coisogenic strain combination was used, the B_6AF_1 (31^-) mice being the recipients of $B_{10}.D_2$/new Sn (31^+) cells and tissue. $B_{10}.D_2$ female skin grafts were rejected by B_6AF_1 *females* in 9 days M.S.T. Thus the 31 specificity of the H2-locus expresses itself as strongly in the B_6AF_1 females as the whole H2-locus. In *males* the rejection occurred at a somewhat later time.

According to the literature multiple injections usually have been more effective in accomplishing graft acceptance [22, 120, 169, 240]. In these experiments the number of injections also proved to be important. Significant prolongation only occurred after more than two i.v. injections of frozen-thawed or viable $B_{10}.D_2$ tissue were given. However no increase in graft acceptance was achieved by increasing the number or dose of inoculations any further.

Even a *second set response* could be abolished by i.v. and i.p. injections of frozen-thawed tissue. Two B_6AF_1 mice accepted a second $B_{10}.D_2$ graft beyond 136 days!

Much effort was directed towards the differentiation between *tolerance* and enhancement. Tolerance has been usually established with i.v. injections of lymphoid cells [95, 120, 169, 178, 240]. Frozen-thawed lymphoid cells alone did not have any significant effect in these studies. The addition of liver cells was necessary for the attainment of more consistent prolongation of graft survival. Viable lymphoid cells however produced immunological unresponsiveness, when more than two injections were used.

The skin grafts in these experiments were usually applied on the first day after the last injection. However a better effect was often noted on the 4th day after the last injection (table 9). These results cannot easily be explained by tolerance. On the contrary, the maximal graft acceptance seems to coincide with maximal antibody production, which can be expected on the fourth day. *Enhancement* then, is the most likely explanation. Repeated i.v. injections were

most successful, which may induce a strong antibody response, as reported by Leskowitz e.a. [161]. He noted that one i.v. inoculation of bovine serum albumen in rabbits resulted in neither antibody formation nor delayed hypersensitivity. An i.v. booster injection on the other hand induced a very good antibody response.

An argument against enhancement might be the evidence that liver extracts have always been less effective in inducing immunity and enhancement [124, 132, 151] than spleen cells, whereas in our experiments the presence of liver in the *frozen-thawed* suspension was prerequisite for a good effect. However, *viable* lymphoid cells were effective without the addition of liver cells. It is possible, that enhancement and tolerance might act together in these experiments. Interesting in this respect is the finding of Dresser [73], who showed that the administration of CBA anti-BCG serum in BCG-tolerant CBA mice did not increase the mechanism of recovery from the state of paralysis, but instead could inhibit this recovery.

Further proof for enhancement in these experiments are the favourable results with *passive transfer* of serum from those B_6AF_1 mice, which were treated with i.v. injections of frozen-thawed $B_{10}.D_2$ tissue. With this serum a significant prolongation of skin graft survival was obtained, which was quite comparable to the prolongation observed in those mice, that produced the serum. The immunological unresponsiveness could thus be transferred by serum. Hence enhancement is a likely mechanism of these phenomena.

5. Summary

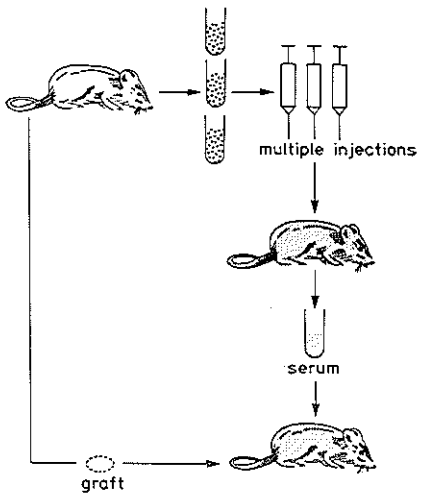
Active enhancement of mouse skin grafts was obtained in a coisogenic strain combination, where the H2-31 specificity appears to form a strong histocompatibility barrier.

Pretreatment of the B_6AF_1 recipients with either frozen-thawed or viable $B_{10}.D_2$ cells resulted in prolonged acceptance of skin grafts, provided that more than 2 injections were given. Even second skin grafts were accepted for a longer period in this experimental model.

Proof for enhancement was provided obtaining a similar effect through transfer of serum and the observation that maximal graft survival occurred in the period of the expected maximal antibody formation.

CHAPTER V

PASSIVE ENHANCEMENT OF SKIN ALLOGRAFTS IN MICE



1. Introduction

After our initial success with *active* enhancement of skin grafts, a model was designed, in which *passive* enhancement of skin grafts could be established [130b]. The mechanism of enhancement can be best studied with passive enhancement, for tolerance may play a role in active enhancement.

In the case of an afferent or efferent mechanism of enhancement it is important to cover all the alloantigens by specific antibodies [71, 189, 192]. Fortunately, greatly simplified experimental models have become available with the development of *congenic strains* of mice, which are virtually identical in genotype, except for an allelic difference at a single histocompatibility locus. Such a combination was used in these experiments, with a single histocompatibility difference at the H2-locus. There are, however, many specificities in the H2-locus and to be able to cover all the foreign alloantigens by antibody, we used combinations where the recipient differed only at one H2-specificity from the donor, namely the *31*-specificity or *32*-specificity (table 1).

2. Material and methods

Animals

B_6AF_1 ($H2^{ab}$) mice ($C_{57}BL/_{6J} \times A/Jax\ F_1$ hybrids) were used as recipients for

tissue or skin of $B_{10}.D_2$/new Sn ($H2^d$), $B_{10}.BR$ ($H2^{bk}$) and ($B_{10}.D_2 \times B_{10}.BR)F_1$ mice. They are coisogenic for respectively the *31*, the *32* specificity and *31*+*32* specificity of the H2-locus. $C_{57}BL/_{10}$ and $(129 \times A)F_1$ mice were recipients for $B_{10}.D_2$ skin grafts in order to test the specificity of the anti-31 serum. $B_{10}.D_2$ mice differ from the $C_{57}BL/_{10}$ mice at the H2-specificities 3, 4, 8, 10, 13, 31. The $B_{10}.D_2$ and $(129 \times A)F_1$ mice are apart from the H2-31 specificity also incompatible at non-H2-loci.

Antiserum

For the acquisition of hyperimmune anti-31 serum, B_6AF_1 mice were repeatedly immunized with viable $B_{10}.D_2$ lymphoid cells for about four months. Seven to twenty days after the last i.p. injection, the mice were bled. The serum was pooled and stored at −20 °C. Anti-32 serum was prepared in B_6AF_1 mice by immunizing them with C_3HK ($H2^k$) lymphoid tissue and Freund's adjuvant for two months. The mice were bled from the retro-orbital sinus. The blood was kept at room temperature until clotting occurred and afterwards at −4 °C overnight for further contraction of the clot.

Titers

Cytotoxic titers up to 1:1000 were measured in the sera by McKenzie [174, 175].

Preparation of the cells

For the preparation of viable cells, spleen and lymphe nodes were collected and treated as described on p. 74.

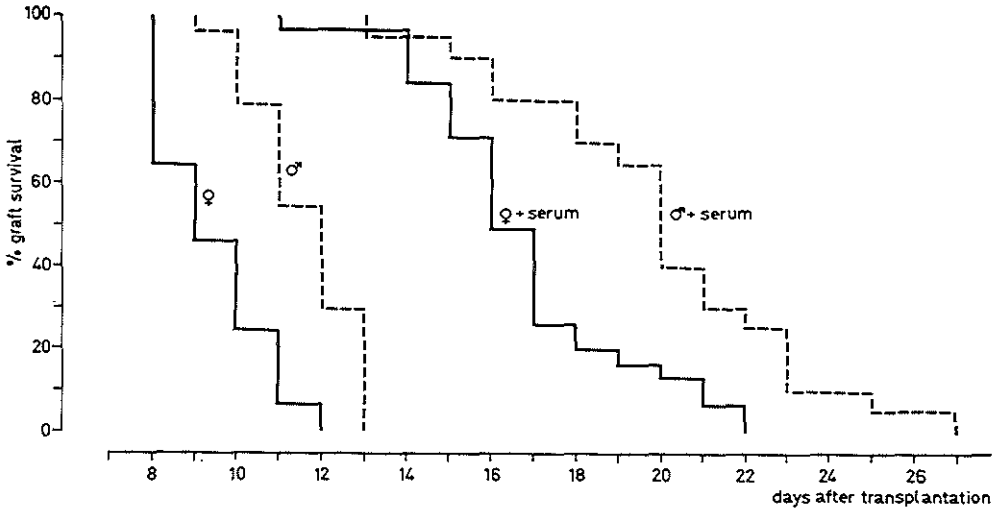
3. Results

The $B_{10}.D_2$ mice only differ from the B_6AF_1 mice at the 31 specificity of the H2-locus. Yet the $B_{10}.D_2$ skin graft survival times on B_6AF_1 females do not differ considerably from those occurring across a H2-locus incompatibility (fig.8).

A weaker immune response of the males became apparent in this combination, as was shown in the previous experiment. Hence male and female mice were used separately in these experiments. Antiserum was injected in amounts of 0.2 ml every other day until the day of rejection unless otherwise stated.

Effect of multiple injections of anti-31 serum

Serum injections of 0.2 ml every other day in 31 female- and 20 male B_6AF_1 recipients, resulted in a significant prolongation of $B_{10}.D_2$ skin graft survival time (fig. 8). By prolonging the survival of skin grafts, a bigger spread in survival



		n	M.S.T.	$P_{Student}$	$P_{Wilcoxon}$
♀	controls	45	9		
♂	controls*	24	11	< 0.0001 *	< 0.0001 *
♀	plus serum	31	16	< 0.0001	< 0.0001
♂	plus serum	20	20	< 0.0001	< 0.0001

* males compared to females

Fig. 8. Effect of anti-31 serum on the survival of $B_{10} \cdot D_2$ ♀ skin grafts to B_6AF_1♀♀ and ♂♂

time was noted. Such a spread can be expected with the use of weak H-loci [121, 123]. Thus the treatment with anti-31 serum seems to reduce the strong H2-31-locus to a weak H-locus, by partially or temporarily eliminating the disparity for the 31 specificity. The magnitude of extension of graft survival by the anti-31 serum is similar in the female and male recipients. This is in contradiction to the opinion of many investigators, who claim that enhancement is easier procured in male recipients.

This experiment depicted in fig. 8 shows that passive enhancement of skin grafts can be obtained in mice. The antisera were usually injected intraperitoneally. Intraperitoneal injections of anti-31 serum proved to be as effective as intravenous injections (table 13).

Table 13. Effect of route of serum injections. $B_{10} \cdot D_2$ skin grafts on B_6AF_1 males and females, treated with anti-31 serum.

sex	route of injection	n	M.S.T.	$\bar{x}$	s.d.	$P_{Student}$	$P_{Wilcoxon}$
♂	i.p.	5	20	20.6	1.82	0.15–0.30	< 0.6
♂	i.v.	5	20	19.0	3.0		
♀	i.p.	7	16	15.6	1.51	0.25–0.50	< 0.9
♀	i.v.	9	17	16.2	2.11		

Effect of one serum injection

It was investigated how long after skin grafting passive enhancement could still be obtained (table 14). Only one injection of antiserum was used. An injection on the second day after transplantation still resulted in optimal prolongation of graft survival. After day 4 no enhancement occurred. Surprisingly, a slightly earlier rejection was noted when the serum was injected on the 7th day after transplantation. One injection of 0.2 ml anti-31 serum appeared to be less effective than injections every other day (table 15). Doses varying from

Table 14. $B_{10} \cdot D_2$ skin graft to B_6AF_1 recipients. Effect of one injection of anti-31 serum (0.20 ml) on different days after skin grafting.

controls	sex	serum injection on day	n	$\bar{x}$	s.d.	$P_{Student}$	$P_{Wilcoxon}$
B_6AF_1	♀	–	45	9	1.32		
B_6AF_1	♂	–	24	11.6	1.21		
	♀	0	18	14.6	1.50	< 0.00001	0.0000000003
	♀	1	10	14.8	2.04	0.00005	0.000002
	♀	2	9	15.0	1.32	0.00001	0.0000008
	♂	4	10	12.8	1.81	< 0.10–0.05	0.025
	♂	5	9	12.7	2.96	0.50–0.20	0.176
	♂	6	7	14.7	4.46	0.20–0.10	0.071
	♂*	7	9	10.3	1.41	0.05–0.02*	0.017*

* shorter survival time than controls B_6AF_1 ♂

Table 15. Effect of different dosages of anti-31 serum on the enhancement of $B_{10}.D_2$ skin grafts in B_6AF_1 recipients

		serum injections							
	sex	1× or multiple	dose/inj.	n	M.S.T.	$\bar{x}$	s.d.	$P_{Student}$	$P_{Wilcoxon}$
controls	♀	–	–	45	9	9.4	1.32		
controls	♂	–	–	24	11	11.6	1.21		
	♀	1×	0.20 ml	24	15	14.7	1.96	< 0.0005	< 0.0000000001
	♀	multiple	0.20 ml	31	16	16.7	2.52	< 0.001–0.0005*	< 0.014*
same	♀	1×	0.05 ml	6	15	14.5	0.84	< 0.0005	< 0.00001
serum	♀	1×	0.20 ml	5	15	15.2	2.59	0.30–0.60**	< 0.6**
same	♂	1×	0.01 ml	5	14	14.0	1.0	0.0025–0.05	< 0.0004
serum	♂	1×	0.10 ml	4	14.5	13.5	3.32	0.40–0.80***	< 0.9***
	♂	1×	1.0 ml	5	14	14.2	1.79	0.40–0.80***	< 0.8***

* compared to the group with one serum injection
** compared to the group with an injection of 0.05 cc serum
*** compared to the group with an injection of 0.01 cc serum

0.01 ml to 1.0 ml in males and 0.05 ml to 0.30 ml in females did not induce different degrees of enhancement.

Apparently only a small dose is sufficient for the attainment of enhancement. In this experiment, the prolongation of graft survival by one injection seems more significant with the use of female recipients. However, the two sera were not part of the same batch.

Specificity of the anti-31 serum (table 16)

The specificity was tested by transferring the serum to $(129 \times A)F_1$ mice and $C_{57}BL/_{10}$ mice, which were subsequently grafted with a $B_{10}.D_2$ skin. The $C_{57}BL/_{10}$ mice are coisogenic with the $B_{10}.D_2$ mice and differ at the H2-allele, including 5 H2-specificities in addition to the 31-specificity. The histo-incompatibility in the combination $B_{10}.D_2 \rightarrow (129 \times A)F_1$ consists of the H2-31-specificity and non-H2-loci. In this last group no significant difference in survival time occurred between the control group and the recipient treated with serum. However, anti-31 serum induced a slight prolongation of graft survival time in the $C_{57}BL/_{10}$ mice. Apparently the monospecific anti-31 serum can also exert a weak enhancing effect on the other 5 H2-specificities.

Table 16. Specificity of anti-31 serum. $B_{10}.D_2$ skin grafts to $C_{57}BL/_{10}$ and $(129 \times A)F_1$ mice.

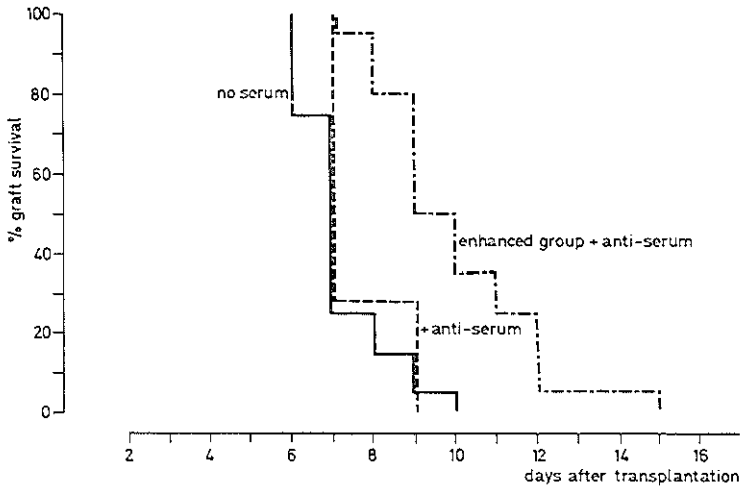
recipient	anti-31 serum	n	M.S.T.	$\bar{x}$	s.d.	$P_{Student}$	$P_{Wilcoxon}$
$C_{57}BL/_{10}$	—	10	9.5	9.7	1.85		
$C_{57}BL/_{10}$	+	9	12	11.5	1.42	0.025	< 0.07
$(129 \times A)F_1$	—	5	12	11.8	1.92		
$(129 \times A)F_1$	+	13	13	12.5	1.56	0.25–0.50	< 0.5

Effect of anti-31 serum in immunized recipients

The anti-31 serum was tested in recipients that were sensitized either by a previous skin graft or by an injection with donor-cells. A *second* skin graft was applied after the wound of the first graft healed, i.e. about 14 days after rejection. The rejection of a second skin graft could not be influenced by antiserum, when the first skin graft was rejected in a normal time. This is in accordance with the findings of other investigators. Enhancement of a second set response has up till now never been reported.

In contrast a group of 20 B_6AF_1 females, which were also treated with anti-31 serum during the first skin graft procedure, rejected a second $B_{10}.D_2$ skin graft at a later time. Thus enhancement of a second skin graft is possible, provided that the anti donor serum is injected not only in the second but also the first skin grafting procedure (fig. 9).

The anti-31 serum was also effective when given together with an otherwise



———— without antiserum
– – – – – with anti-31 serum.
—·—·— with anti-31 serum. The recipients were also treated with anti-31 serum during the first skin graft procedure.

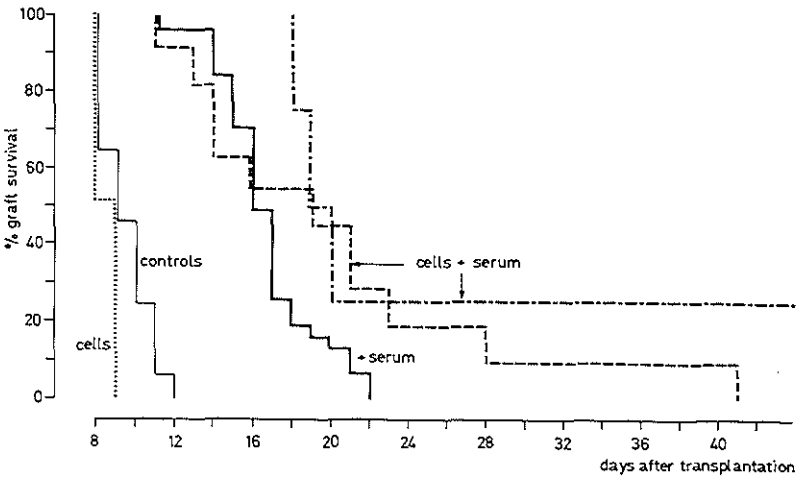
	n	M.S.T.	s.d.	$P_{Student}$	$P_{Wilcoxon}$
————	20	7	1.24		
– – – – –	11	7	0.93		
—·—·—	20	9.5	1.83	< 0.0005	< 0.0001

Fig. 9. Effect of anti-31 serum on the survival of second $B_{10}.D_2$ skin grafts.

sensitizing i.v. injection of *viable cells* (fig. 10). One injection of $18–30 \times 10^6$ viable $B_{10}.D_2$ lymphoid cells, given a few hours before skin grafting, resulted in a slightly accelerated rejection of $B_{10}.D_2$ skin grafts. When however anti-31 serum was added to the cell suspension and subsequently injected every other day, prolongation occurred. One $B_{10}.D_2$ skin was even tolerated for more than 150 days, after an i.v. injection of 42×10^6 viable $B_{10}.D_2$ spleen and lymphe node cells was given. With the two higher doses the prolongation of graft survival was more pronounced than with anti-31 serum alone. Apparently a mixture of donor antigens and anti-donor antibodies may also induce enhancement and even at a much more significant level when the appropriate cell dosage is used.

Effect of irradiation in addition to serum treatment

Antiserum injections used in combination with irradiation, caused a very long prolongation of graft survival time. B_6AF_1 male mice were irradiated with 400 R total body dose, 24 hours before skin grafting. Anti-31 serum was given every other day. The control group, which was only irradiated, did reject the $B_{10}.D_2$ skin grafts in 16 days M.S.T. The M.S.T. in the experimental group



		n	M.S.T.	s.d.	$P_{Student}$	$P_{Wilcoxon}$
———	control B_6AF_1	45	9	1.32		
.....	$18–30 \times 10^6$ cells	8	8.5	0.53		
———	+ anti-31 serum	31	16	2.52	$\leqslant 0.00001$	< 0.0001
	1×10^6 cells + A.S.*	11	16	3.80	0.00006	< 0.0001
	18×10^6 cells + A.S.*	10	16.5	2.79	< 0.00001	0.0001
– – – – –	30×10^6 cells					
	+ anti-31 serum	11	19	8.57	0.005	0.0001
—·—·—	42×10^6 cells					
	+ anti-31 serum	4	19.5	25.51	0.20–0.10	0.0005

Fig. 10. $B_{10}.D_2$ skin grafts to B_6AF_1 ♀
Effect of – anti-31 serum
– anti-31 serum + viable donor cells
– viable donor cells

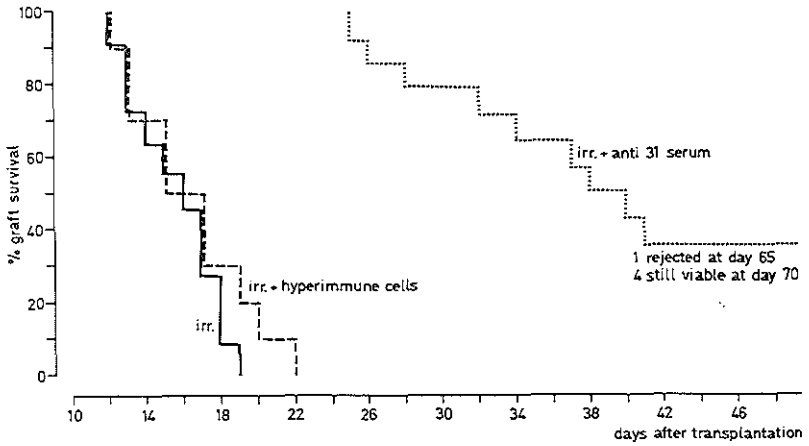
* Not depicted in the figure.

was 39 days, while 4 skin grafts were tolerated for more than 70 days. Enhancement did not manifest itself, when the irradiated B_6AF_1 mice were injected on the day of transplantation with 100×10^6 viable spleen and lymphe node cells, collected from those hyperimmunized B_6AF_1 mice, which were the source of the enhancing anti-31 serum (fig. 11).

Thus enhancement of skin grafts is much more pronounced when the treatment with anti donor serum is combined with irradiation.

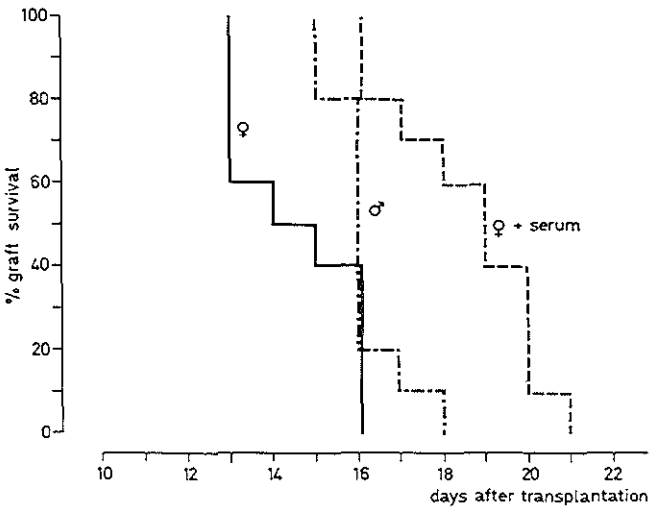
Effect of multiple injections of hyperimmune anti-32 serum

In addition to the H2-31-specificity, the 32-specificity of the H2-locus was also tested. For this purpose, $B_{10}.BR$ (32^+) skin grafts were applied to B_6AF_1 (32^-) recipients. This again is a monspecific coisogenic combination, where the recipient only differs from the donor at the H2-32-specificity (table 6). The rejection reaction was much weaker in this combination than was the case with the H2-31 barrier (fig. 12). Again a difference existed between the im-



		n	M.S.T.	s.d.	P_{Student}	P_{Wilcoxon}
	controls	45	9			
———	irr.	11	16	2.38	0.0002	0.00002
– – – – –	irr. + hyperimmune cells	10	16	3.30	0.002–0.001	0.00005
·····	irr. + anti-31 serum	14	39	18.35	< 0.00001	< 0.0000001

Fig. 11. $B_{10}.D_2$ ♀ skin grafts to irradiated B_6AF_1 ♀ mice.
Effect of – anti-31 serum
– hyperimmune B_6AF_1 lymphoid cells



		n	$\bar{x}$	s.d.	$P_{\text{Student Welch}}$	P_{Wilcoxon}
———	controls B_6AF_1 ♀	10	14.5	1.43		
—·—·—	controls B_6AF_1 ♂	10	16.1	0.88	0.01 *	0.01 *
– – – – –	B_6AF_1 ♀ treated with anti-32 serum	10	18.6	1.78	0.0002	0.0002

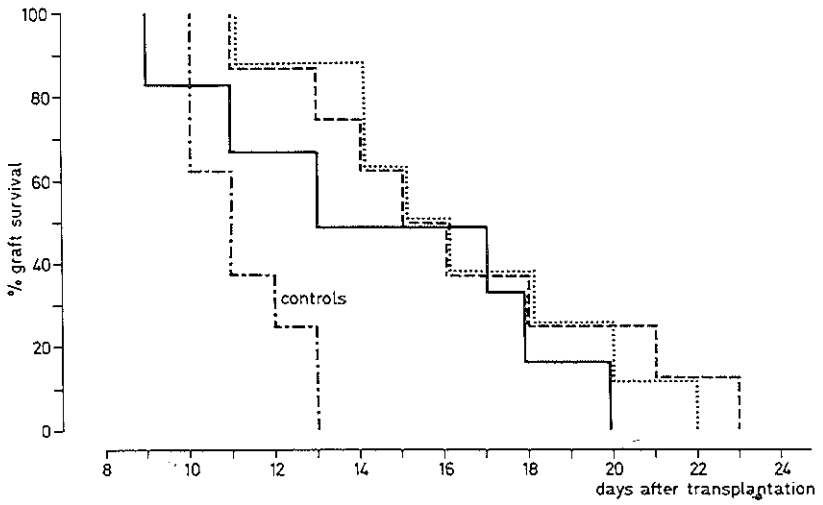
Fig. 12. $B_{10}B_R$ skin grafts to B_6AF_1 mice. Effect of anti-*32* serum.
Antigenic disparity: H_2-32 specifity.

* compared to ♀ controls

mune response of males and females ($p < 0.01$). Multiple injections of 0.2 ml hyperimmune anti-32 serum in B_6AF_1 mice, induced a significant prolongation of B_{10}.BR skin graft survival. Enhancement of skin grafts then is feasible with anti- 31 and -32 serum in host-donor combinations differing for the H2-31- and H2-32-specificity respectively.

H2-31- and H2-32-specificity

In this experiment anti-31 and anti-32 serum were tested in a bi-specific coisogenic combination. ($B_{10}.D_2 \times B_{10}.BR)F_1$ (31^+, 32^+) mice were the source of the donor skin for the B_6AF_1 (31^-, 32^-) recipients. In this combination, the recipients lack the 31 and 32 specificity of the H2-locus, which are present in the donor strain. The B_6AF_1-recipients were either treated with anti-31 serum, anti-32 serum or anti-31 and anti-32 serum together (fig. 13). Surprisingly, significant extension of graft survival occurred with either the anti-31 or the anti-32 serum alone. The two sera, administered simultaneously, were not more effective than either of them alone. Thus no additive effect occurred of the two enhancing sera.



		n	M.S.T.	s.d.	$P_{Student}$	$P_{Wilcoxon}$
—·—·—	controls	8	11	1.38		
– – – –	anti-31 serum	8	15.5	4.07	0.01–0.002	0.0025
·····	anti-32 serum	8	15.5	3.58	0.01–0.002	0.0020
———	anti-31+32 serum	6	15	4.32	0.20–0.10	0.08

Fig. 13. $B_{10}.D_2 \times B_{10}BR$ skin grafts to B_6AF_1♂. Effect of anti-31, anti-32 and anti-*31+32* serum. Antigenic Disparity: H_2-31+32 specificities.

4. Discussion

In the present experiments, in which mice coisogenic for the *31* and *32* alloantigenic specificity of the H2-locus were used, enhancement of skin grafts was achieved by passive transfer of hyperimmune anti-donor serum. A prolongation in M.T.S. of $B_{10}.D_2$ skin grafts of 9 days occurred in the male B_6AF_1 recipients, which were injected with antiserum, directed against the H2-31 specificity. The 31-specificity has all the characteristics of the strong H2-histocompatibility locus as far as the rejection of skin grafts is concerned. This is, however, only the case, when females are used. The female B_6AF_1 recipients rejected the $B_{10}.D_2$ donor skin graft earlier than the males. Females possess a stronger reacting immune system [165], but this has not been reported with skin grafts across the H2-barrier [16], except for thymectomised recipients [10a]. In these experimental groups, where such a clear difference in immune reactivity exists between females and males, one would expect a much stronger enhancement in males, but this was not the case.

The prolongation of skin graft survival by the action of anti-donor serum in such a simple coisogenic strain combination, provides an excellent model for the study of passive immunological enhancement.

The 32-specificity of the *D-end* of the H2-locus appeared to be weaker in antigenic strength than the 31-specificity (*K-end*). The difference in immune response between male and female B_6AF_1 mice was in this case again apparent. Passive enhancement of $B_{10}.B_R$ skin grafts by the transfer of monospecific anti-32 serum could be obtained in B_6AF_1 recipients. The anti-31 serum as well as the anti-32 serum induced enhancement of $(B_{10}.D_2 \times B_{10}B_R)F_1$ (31^+, 32^+) skin grafts in B_6AF_1 mice.

Surprisingly no better enhancement was attained with the combined use of anti-31 and anti-32 serum. Thus, monospecific antisera could in this experiment produce enhancement in a bi-specific coisogenic combination. This is in contradiction to the theory of Möller [189] that for a good immune inhibition, the enhancing antibodies should be directed against all the antigens of the donor. It might of course be possible that our antisera are not entirely monospecific but cross reacting with certain antigens. Our experiments prove that the anti-31 serum could even induce a slight prolongation of graft survival in a model, where the recipients did not only lack the H2-31-specificity, but also 5 other H2-specificities. No effect was noted on the survival of $B_{10}.D_2$ skin grafts in $(129 \times A)F_1$ mice, which lack the H2-31-specificity in addition to non-H2-loci.

Thus the monospecific anti 31-antibodies still have an enhancing effect in the presence of an incompatibility at six H2-specificities, but none when also non-H2 loci are involved. It has always been a controversial point, whether such antibodies are effective at the whole cell surface, or at the individual

antigenic determinants [71, 116]. The present findings suggest that the anti-31 antibodies can execute their enhancing effect because the six antigenic determinants are close together on the cell surface or because they are similar in structure. A similar phenomenon might be the finding of Greenbury and Moore [97] who reported the immunosuppressive effect of anti-Forsmann antibodies on the humoral response to the red cells in rabbits. To this phenomenon might also be related the naturally occurring protection against Rh immunization, afforded by A.B.O. incompatibility between mother and foetus.

Little information is as yet available about the best time to administer the antiserum. Kaliss [137] was able to enhance tumor grafts up until ten days after the injection of the tumor cells. Dixon [71] reported the suppression of antibody synthesis in rabbits by anti Keyhole Limpet Hemocyanin (KLH) serum given 6–8 days after injection with KLH antigen. In our experiments one injection of anti-31 serum on the fourth day after transplantation could still provoke prolongation of skin graft survival time. On the fourth day after skin grafting the allo-antigen will already be recognized and antibody production is at this time usually at its peak. Yet on this day an antiserum injection is still effective. Thus the mechanism of enhancement is probably not purely afferent and the possibilities of a central or efferent inhibition of the rejection reaction are still left.

Different dosages are reported as the best way to induce passive enhancement. Either small [139, 190] or large [32, 206] doses were needed. Möller [190] even noted two different effects of anti-donor serum. With the same dosage of antiserum he obtained either enhancement or inhibition of tumor growth in mice, depending on the kind of tumor. In our experiments single injections of 0.05 or 0.01 ml antiserum were only slightly less effective than an injection of 1.0 ml. Hence even a small amount of antibody is sufficient to put the enhancing mechanism into action.

In clinical transplantation several immunosuppressive reagents are used together to deal with the rejection reaction. It was therefore of interest to study the effect of our antiserum in combination with immunosuppressive treatment. *Irradiation* was used for immunosuppression, which appeared to work synergistically with the anti-31 serum. $B_{10}.D_2$ skin grafts enjoyed a very prolonged survival on irradiated B_6AF_1 mice, which were treated with antiserum. Antiserum producing lymphoid cells were in this model not effective at all. By inhibiting the immune response in the early phase with irradiation, the antiserum apparently has a better opportunity to induce its enhancing effect. Irradiation might give the antibodies the chance to exert their enhancing effect before the lymphoid cells of the recipient are recovered and finally enter the skin graft.

It has in general been impossible to induce enhancement in previously sensitized animals. Also in our studies a *second* skin graft could not be enhanced

by antiserum. However, when the recipients were previously treated with anti-31 serum during the first skin graft period, a second $B_{10}.D_2$ skin graft enjoyed a prolonged survival. Apparently enhancing antibodies affect the immune mechanism in such a way, that enhancement may again occur with a second skin graft. This is very suggestive for an enhancing activity at the central level, for stimulation of the immune mechanism did already take place during the first skin graft period. The enhancing anti-31 antibodies could also counteract the effect of an otherwise sensitizing injection of viable $B_{10}.D_2$ lymphoid cells and even cause a stronger enhancement than with serum alone. In one case the $B_{10}.D_2$ graft was not rejected at all!

Thus sensitization by lymphoid cells does not occur when the cells are mixed with the anti-31 serum. This strongly suggests that the cellular response is blocked at the peripheral level by the antiserum. The humoral response then might be left, which can sustain the formation of enhancing antibodies, whereas the originally injected antibodies are already destructed [81]. The effectiveness of antigen-antibody precipitates in preventing the immune reaction has also been described for other experimental models [9, 116, 270, 278, 279].

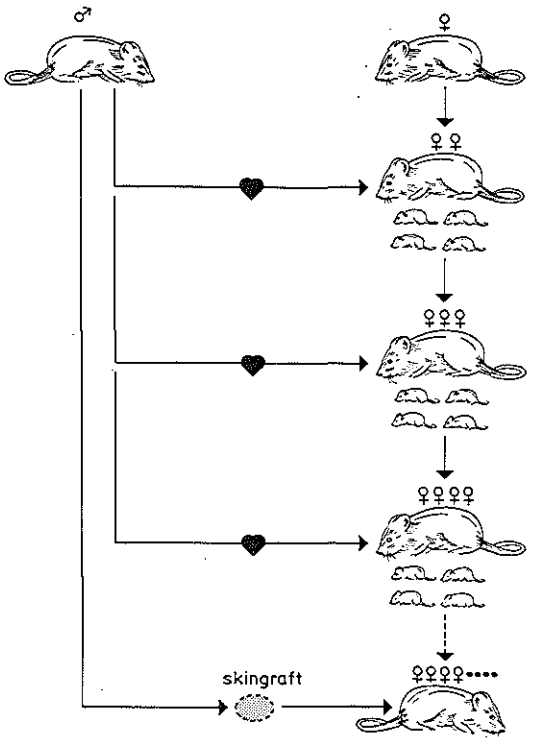
5. Summary

Passive enhancement of skin allografts was achieved in mice. Several findings in these experiments give information about the mechanism of enhancement.

Firstly, enhancement could still be passively obtained up to 4 days after transplantation. This excludes the possibility of an afferent mechanism. Secondly, enhancement of a second skin graft appeared to be possible, provided that the recipients were also injected with antiserum during the first skin graft procedure. A central blockage of the immune response can be the only explanation for this finding.

Furthermore the most significant enhancement was obtained by a combination of anti-31 serum with either irradiation or the administration of viable donor cells. Donor antigens as well as irradiation affect the immune response at the central level. Thus the present experiments manifest the possibility of enhancement of skin grafts and offer speculations about its mechanism.

ENHANCEMENT AND ACCELERATED REJECTION OF MALE SKIN GRAFTS IN ISOGENIC POSTPARTUM FEMALE MICE, AS RELATED TO THE BREYERE AND BARRETT PHENOMENON



♀♀ = first litter
♀♀♀♀●●● = multipara with unknown number of pregnancies

1. Introduction

Breyere and Barrett described in 1960 [35] a decreased resistance of postpartum females to tumor grafts of the male strain with which they had been bred. This effect increased with parity and also occurred with male skin grafts [36, 37, 38, 39, 40, 221, 300]. Some investigators related this phenomenon to a state of tolerance, induced by the continuous contact of the mother with the antigens of the conceptus [37, 38, 39, 40, 221]. An argument against this theory is the demonstration in multiparae of humoral [147, 148, 149, 118] and

cellular [259] immunity against the antigens of the male strain. This suggests that enhancement is the causative mechanism.

The purpose of the present experiments was to test whether enhancement might indeed be the cause of the Breyere and Barrett phenomenon.

Multiparous female mice were bred with males of the same strain. Thus the only antigenic difference was determined by the *HY-locus*. This locus was discovered by Eichwald and Silmser in 1955 [77]. The rejection by females of an isogenic male skin will occur in many inbred strains of mice. The HY-locus is strongly expressed in the $C_{57}BL$/mice. Here the antigen determined by the HY-locus is as potent as the antigens of the other histocompatibility loci in the mouse, except for the H2-locus [25, 26, 159, 285]. It has always been assumed that this male antigen was determined by the Y-chromosome [77, 25, 26, 219, 241]. There is however also some evidence for an autosomal determination of the HY-locus [285, 80].

2. Material and methods

Animals

The following strains were used: $C_{57}BL/_{10}$, $C_{57}BL/_{6}$, $B_{10}.D_2$/new, $B_{10}.129$ (21 M). The retired breeders were not older than 1 year. The number of their pregnancies was unknown. Virgins were only used when 8 weeks or older. The multiparae were divided into many different groups, according to the shipments from Jackson Laboratory, Bar Harbor. The number of pregnancies of the individual animals has not been recorded. They were guaranteed to have had several litters before being shipped.

Serum

The mice were bled from the retro-orbital sinus of the eye. The blood was allowed to clot at room temperature and kept overnight at +4 °C. The serum was pooled and stored at −20 °C. The PVP-method of Stimpfling [268] was used to detect haemagglutinating antibodies and the test of Boyse, Old and Chouroulinkov [29] for cytotoxic antibodies.

Skin grafts

These were applied following the technique of Billingham and Medawar [24].

Parabiosis

Parabiosis was performed by the technique of Sauerbruch and Heyde [237], joining the two animals' peritoneal cavities by suturing the peritoneum and by joining the muscles and skin from ear to tail. 5–0 catgut was used for the peritoneum and muscles and 4–0 silk for the skin. To avoid the post-operative complication of separation of the two animals, interrupted sutures were used

Fig. 14. A multiparous $C_{57}BL/_{10}$ mouse in parabiosis with a $C_{57}BL/_{10}$ virgin.

for the skin and the scapulae were joined with a silk suture. Only the couples that remained in good condition were used in the experiments [fig. 14). Surgical separation was a simple and fast procedure. The mice tolerated the separation with subsequent skin grafting without difficulty.

3. Results

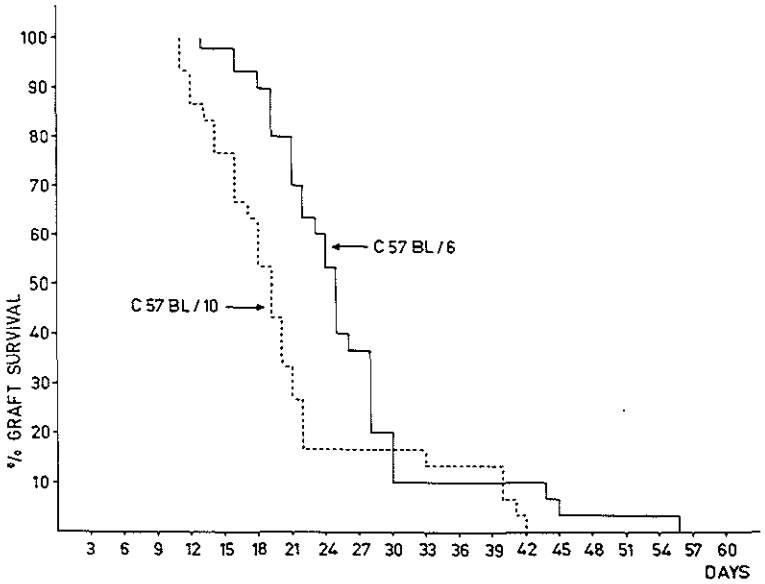
Controls

30 $C_{57}BL/_{10}$ male skin grafts displayed a rather short survival time on $C_{57}BL/_{10}$ females, with a Mean Survival Time ($\bar{x}$) of 21.2 days, s.d. 9.58. As is shown in fig. 15, 30 female $C_{57}BL/_{6}$ mice rejected an isogenic male skin graft later with a $\bar{x}$ of 26 days, s.d. 8.87 and a survival time ranging from 19–57 days. A large spread exists in the survival times of the individual grafts, as is to be expected in the presence of weak antigenic differences [123].

Experimental groups

According to the literature, multiparous females will reject isogenic male skin grafts much later or not at all (fig. 16+17). This however was not always the case in our experimental 209 multiparous $C_{57}BL/_{10}$ and $C_{57}BL/_{6}$ females. Table 17 shows the results with the various multipara groups.

Many isogenic male skin grafts did not enjoy an extended survival time, but instead were rejected in either a normal time or even in an accelerated way



		n	$\bar{x}$	s.d.
———	$C_{57}BL/_6$	30	26.0	8.87
- - - - -	$C_{57}BL/_{10}$	30	21.2	9.58

Fig. 15. Isogenic male skin grafts to female $C_{57}BL/_6$ and $C_{57}BL/_{10}$ mice.

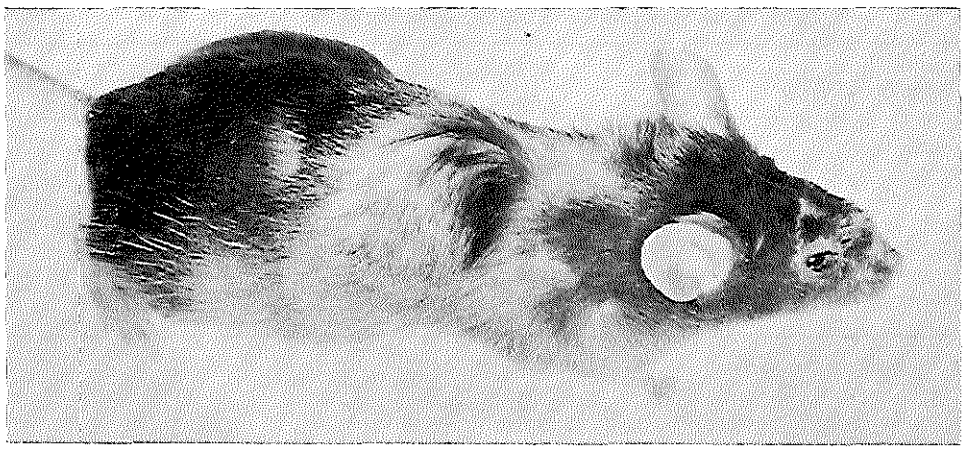


Fig. 16. $C_{57}BL/_6$ male skin graft 60th day after transplantation to $C_{57}BL/_6$ multipara.

(table 17). The total number of 150 $C_{57}BL/_6$ multiparae did not reject an isogenic male skin graft significantly later. A striking effect was noted with some experimental groups. These multipara groups (Ib and IIb) displayed an accelerated rejection (fig. 18).

Also $B_{10}.D_2$ retired breeders (fig. 19) rejected $B_{10}.D_2$ male skin grafts earlier than $B_{10}.D_2$ virgins. No correlation was noted between the period of time that the skin grafts were applied after the arrival of the mice in the laboratory and the time of rejection.

Table 17. Isogenic male skin grafts to female $C_{57}BL/_6$ (I) and $C_{57}BL/_{10}$ (II) multiparae. Groups I and II were divided in sub-groups, respectively I*a–k* and II*a–e* according to their time of arrival from Bar Harbor.

$C_{57}BL/_6$ male skin grafts to $C_{57}BL/_6$ multiparae

group I controls: (virgins)		*n* 30	$\bar{x}$ (days) 26.0	s.d. (days) 8.87	$P_{Student}$ –	$P_{Wilcoxon}$ –
multiparae						
I A	I *b*	9	47.1	22.04	*0.05–0.02*	*0.013*
	I *a*	9	40.1	23.62	0.20–0.10	0.17
	I *k*	5	35.0	0.00	*< 0.001*	*0.005*
	I *c*	18	31.3	9.88	0.10–0.05	*0.006*
	I *f*	10	30.5	5.23	0.10–0.05	*0.004*
	I *h*	15	29.3	11.23	0.50–0.20	0.45
	I *j*	27	27.9	11.34	0.50–0.20	0.39
	I *i*	24	26.8	8.96	> 0.50	0.42
I B *	I *d*	11	19.6	5.07	*0.01–0.002*	*0.01*
	I *e*	16	20	6.93	*0.05–0.02*	0.085
	I *g*	6	23.5	6.47	0.50–0.20	0.80
I A+B	I *a* t/m *k*	150	29.0	12.89	0.20–0.10	0.16

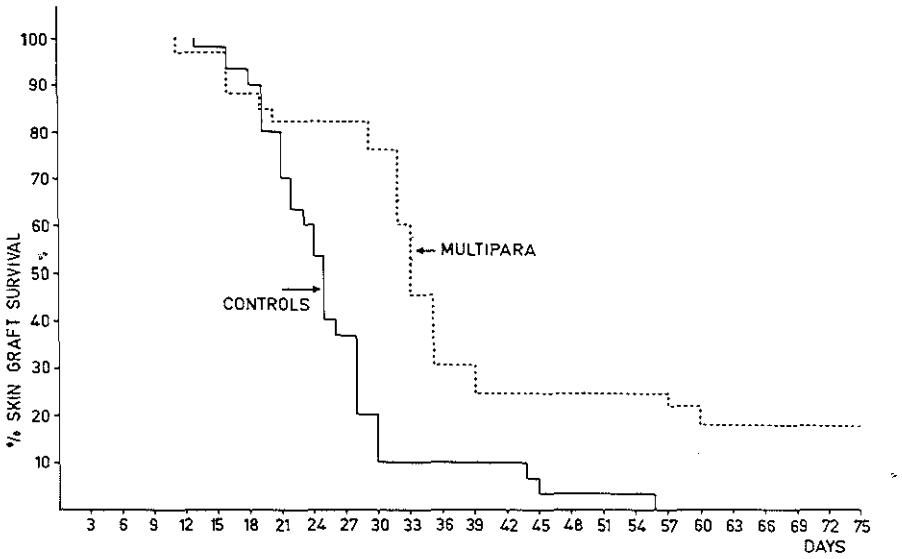
$C_{57}BL/_{10}$ male skin grafts to $C_{57}BL/_{10}$ multiparae

group II controls: (virgins)		*n* 30	$\bar{x}$ (days) 21.2	s.d. (days) ± 9.58	$P_{Student}$ –	$P_{Wilcoxon}$ –
multiparae						
II A	II *a*	9	39.3	24.08	0.10–0.05	*0.016*
	II *b*	8	23.0	5.61	> 0.50	0.20
	II *c*	11	23.9	5.49	0.50–0.20	*0.034*
II B *	II *d*	15	16.7	3.06	*0.05–0.02*	0.30
	II *e*	16	20.6	7.64	> 0.50	0.90
II A+B	II *a* t/m *e*	59	23.4	12.62	0.50–0.20	0.20

* accelerated rejection

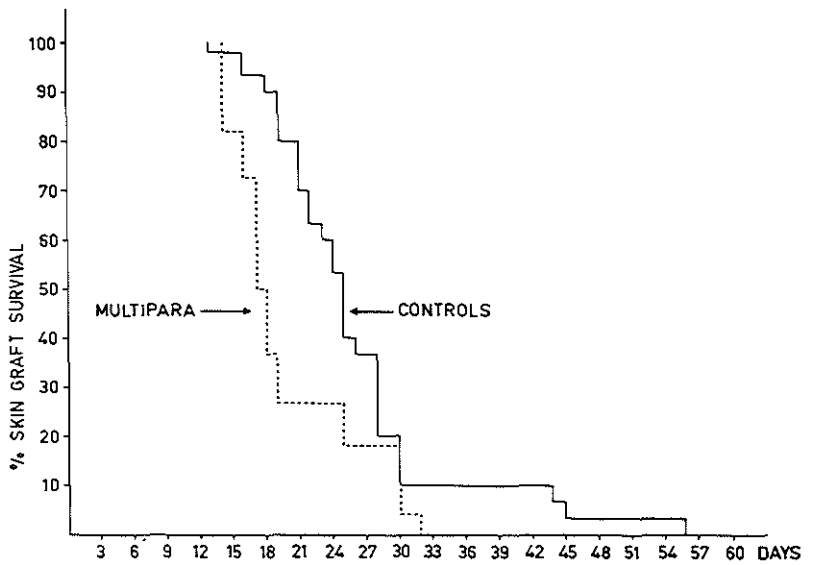
Second set response

The few multiparous $C_{57}BL/_6$ mice which accepted male skin grafts for a prolonged period were tested with a second skin graft. The first male skin grafts were excised when still 100% viable for more than 30 days after grafting. Fig. 20 shows that the second skin grafts also survived longer on these multiparae. A few multiparae received a third graft. In these animals the rejection occurred at a later time too.



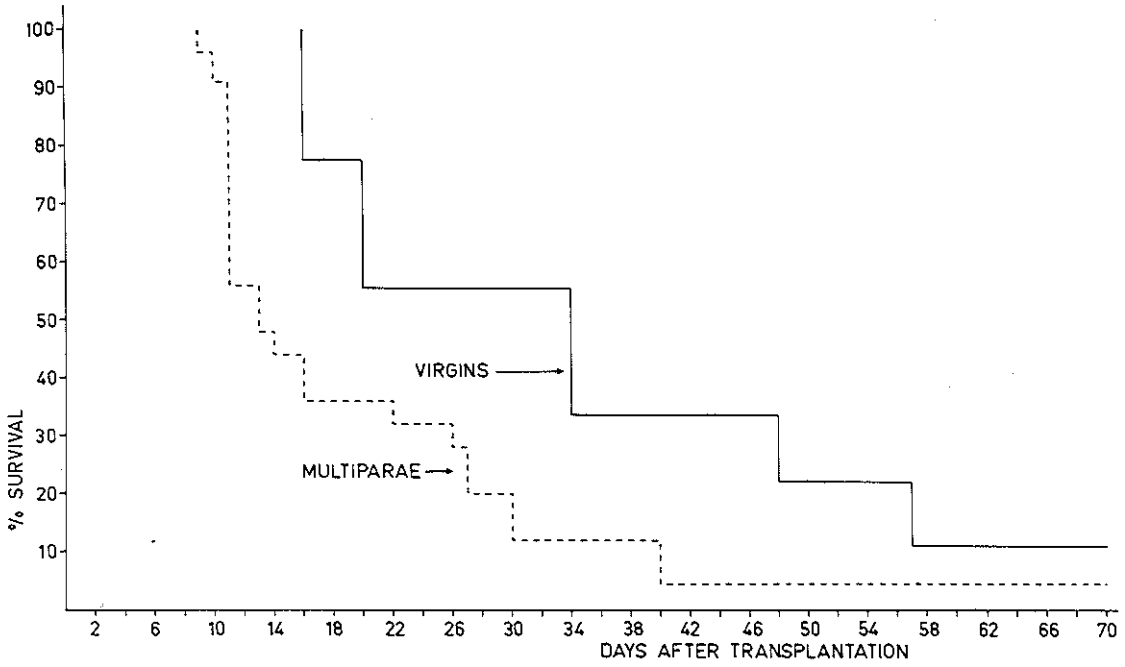
		n	$\bar{x}$	s.d.	P_{Wilcoxon}
———	controls (virgins)	30	26.0	8.87	
– – – –	multiparae	33	38.3	17.67	< 0.001

Fig. 17. Isogenic male skin grafts to $C_{57}BL/_6$ females.



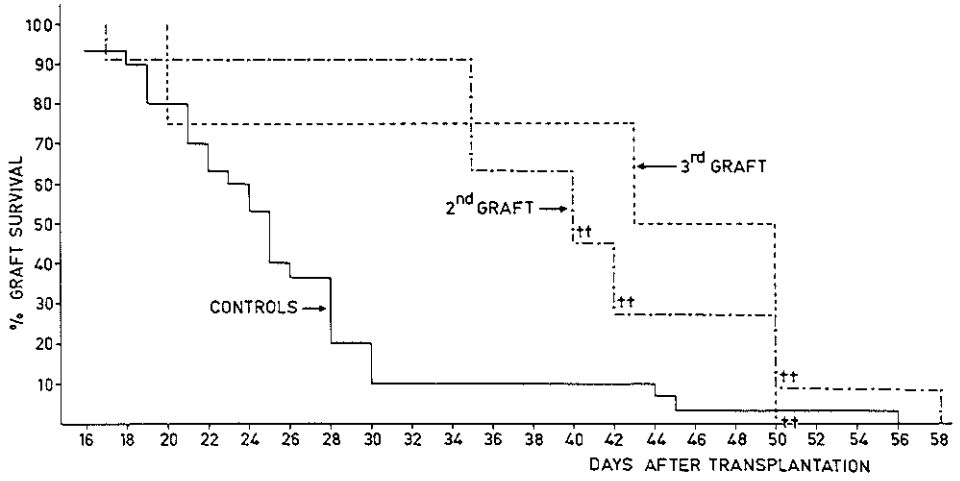
		n	$\bar{x}$	s.d.	P_{Wilcoxon}
———	controls (virgins)	30	26	8.87	
– – – – –	multiparae	11	19.6	5.07	0.01–0.02

Fig. 18. Isogenic male skin grafts to $C_{57}BL/_6$ females.



		n	$\bar{x}$	s.d.	$P_{Student}$	$P_{Wilcoxon}$
———	virgins	9	35	19.53		
– – – –	multiparae	25	20.1	14.07	0.10–0.05	< 0.01

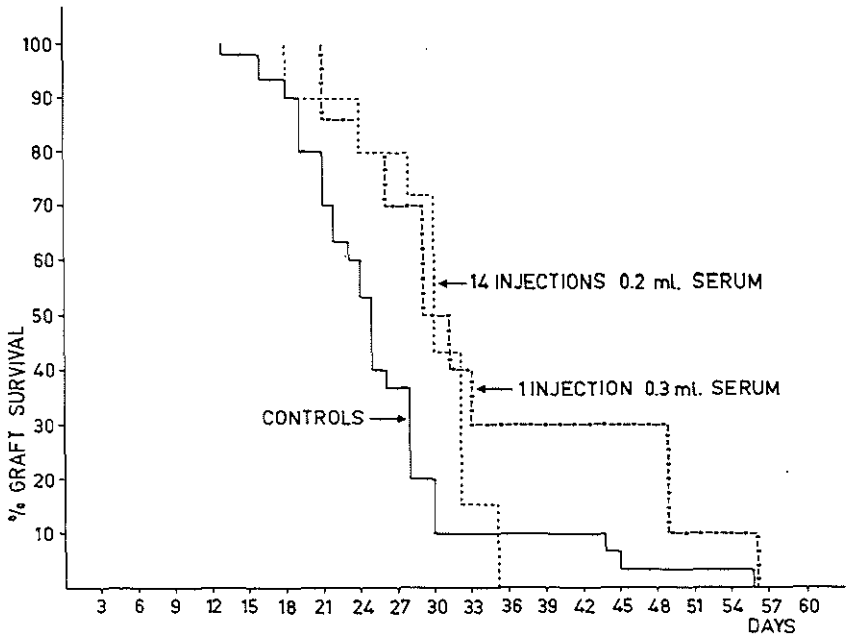
Fig. 19. Isogenic male skin grafts to $B_{10}.D_2$ virgins and multiparae.



		n	$\bar{x}$	s.d.	$P_{Student}$	$P_{Wilcoxon}$
———	controls (virgins)	30	26.0	8.87		
—·—·—	I 2nd skin graft	11	37.2	10.27	< 0.01	0.002
– – – – –	II 3rd skin graft	4	33.5	9.00	0.20	0.10

of group I 6 were killed with 100% viable skin grafts
of group II 2 were killed with 100% viable skin grafts

Fig. 20. 2nd and 3rd male isogenic skin grafts on multiparous $C_{57}BL/_6$ females after excision of the first skin grafts.



		n	$\bar{x}$	s.d.	P_{Wilcoxon}
———	controls	30	26.0	8.87	
- - - - -	14 injections	7	29.0	4.42	0.02
—·—·—	1 injection	10	34.4	12.53	0.02

Fig. 21. Passive enhancement by passive transfer of multipara serum to $C_{57}BL/_6$ virgins.

Passive transfer

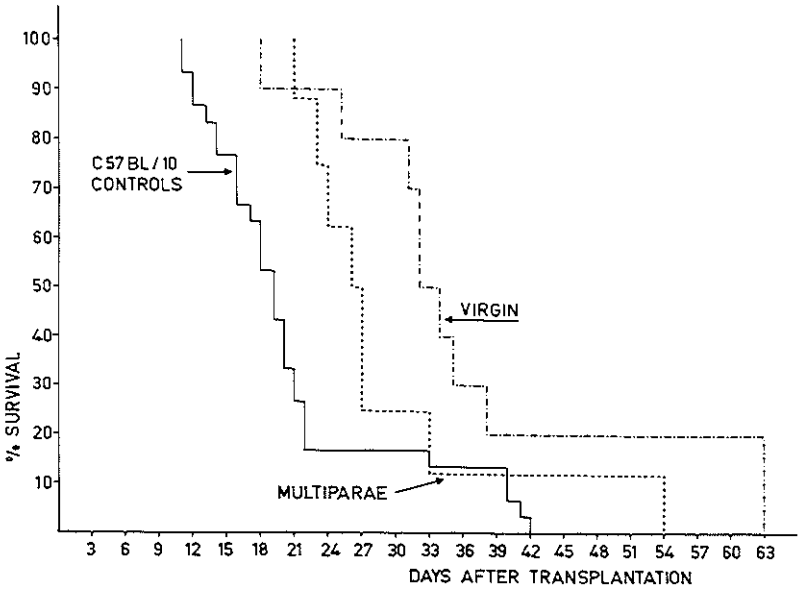
Serum obtained from multiparous $C_{57}BL/_6$ mice, was injected in amounts of 0.2 ml i.p. in isogenic virgins (fig. 21). One group received one injection. Another group received 14 injections, one every other day. The isogenic male skin grafts enjoyed an extended survival in both groups. Apparently one injection of antiserum is in this experiment as effective as multiple injections.

Serology

The haemagglutinin and cytotoxin titers were tested in the pooled sera of several multipara groups. No haemagglutining or cytotoxic antibodies could be detected.

Parabiosis

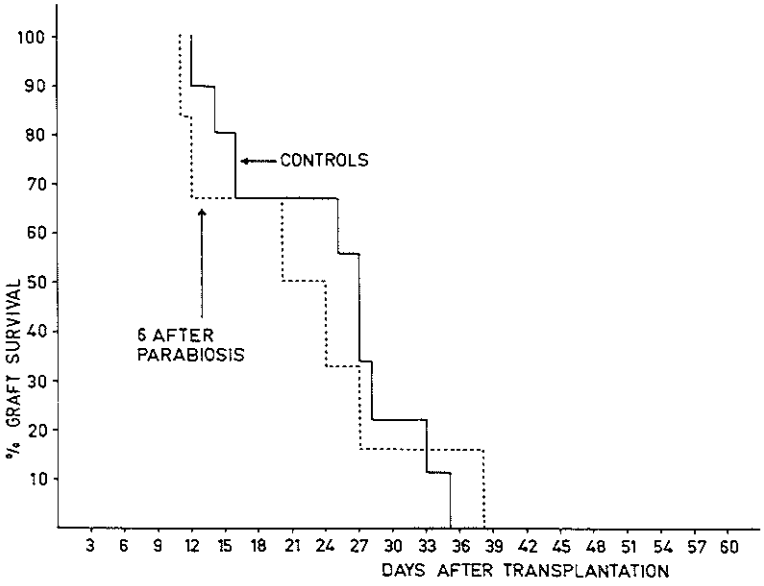
$C_{57}BL/_{10}$ multiparae were joined in parabiosis with $C_{57}BL/_{10}$ virgins. After one month of parabiosis they were separated and on the same day grafted with



		n	M.S.T.	s.d.	$P_{Student}$	$P_{Wilcoxon}$
———	$C_{57}BL/_{10}$ controls	30	21.2	9.58		
—·—·—	$C_{57}BL/_{10}$ virgins*	10	35.8	12.63	0.01–0.002	0.003
– – – – –	$C_{57}BL/_{10}$ multiparae*	8	29.0	10.90	0.10–0.05	0.01

* after parabiosis

Fig. 22. Enhancement of male skin grafts after parabiosis of virgin and multipara.



		n	M.S.T.
———	controls	10	27
– – – – –	after parabiosis of multiparous and virgin $C_{57}BL/_{10}$ mice	6	24

Fig. 23. B_{10}.129 21 M skin grafts on $C_{57}BL/_{10}$ mice.

a $C_{57}BL/_{10}$ male skin. Only animals without post-operative complications were used in these experiments.

$C_{57}BL/_{10}$ virgins, after parabiosis with multiparae, accepted male skin grafts for a much longer period than virgin controls, as is shown in fig. 22. A normal rejection of skin grafts from $B_{10}.129$ (21 M) mice occurred in $C_{57}BL/_{10}$ virgins, which had been in parabiosis with isogenic multiparous females for four weeks (fig. 23).

4. Discussion

The purpose of these experiments was to distinguish between enhancement and tolerance in the phenomenon of *Breyere* and *Barrett*, i.e. the acceptance of male skin- and tumor-grafts by the multiparae with which they had been bred [35, 36].

Tolerance has predominantly been accepted as the mechanism of this phenomenon, as no proof for enhancement existed. A naturally occurring coisogenic intrastrain combination, differing in the *HY-locus* served as a model for our experiments. This locus manifests itself as a rather strong locus in the $C_{57}BL$ mice.

$C_{57}BL$ female *multiparae*, mated within the strain, in some instances tolerated the isogenic male skin grafts for a longer time than nulliparae, as was expected from the experience of Breyere and Barrett.

In contrast, however, some groups displayed an accelerated rejection of the male skin grafts, which suggests that they were sensitized in the course of their pregnancies. Thus a variety of rejection patterns was exhibited by the various multipara groups as they were purchased from Bar Harbor, which manifested itself in both increased and decreased male skin graft survivals. It might be-possible that the contact with foetal antigens either induces a sensitization or an impaired immunological response. Enhancement then seems the most likely explanation for the Breyere and Barrett phenomenon, because in contrast to tolerance, both immunity and enhancement depend on the presence of the immune response and not on its absence.

The results with the *passive transfer* of serum from multiparae furnish additional proof for enhancement. This test, which is crucial for enhancement, was contrived by injections of serum from multiparae into isogenic virgins and by *parabiosis* of a multipara and a nullipara. In both cases a prolongation of male skin graft survival occurred. By parabiosis, however, a much longer prolongation was obtained, probably because of the more continuous and complete exchange of serum between the partners. In such an isogenic combination a common blood circulation is established within a few days after parabiosis is performed [171]. Transfer of cellular immunity is then also possible and one might expect a second set response in the virgins. Instead, a reduction in

immunological responsiveness occurred in the virgins after parabiosis with multiparae. Apparently, the enhancing antibodies, are able to inhibit the existing cellular immunity. The immunological specificity of this effect was proven by the normal rejection of $B_{10}.129$ (21 M) skin grafts by $C_{57}BL/_{10}$ nulliparae, which had been in parabiosis with multiparae. In this combination an incompatibility exists at the weak H4-locus, which was used to reveal any non specific effect which might be more obvious in respect of a weak histocompatibility antigen.

The observation in our experiments of both prolonged survival and accelerated rejection suggests that virtually every multipara is sensitized by foetal antigens. Indeed haemagglutinins and leukagglutinins against H2-antigens of the male partner have been also demonstrated in the serum of postpartum female mice [147, 148, 149, 118]. In addition to humoral immunity, cellular immunity also seems to exist in the postpartum female. This has been reported by Sören [259]. He demonstrated that lymphoid cells of multiparae evoke an intensified graft versus host reaction in their newborn offspring.

The expected second set response to a male skin graft in the multiparae might be inhibited by enhancing antibodies. Enhancement then appears to be responsible for prolongation of graft survival and for the normal rejection times in the bulk of the multipara groups. Indeed second and third male skin grafts were also accepted for a longer time, when grafted on to selected multiparae which already displayed enhancement.

Thus every pregnancy induces a sensitization towards the foetal antigens, which in most cases is masked by the occurrence of enhancing antibodies. In such a way the foetus itself can also be protected from immunological rejection by cells. The foetus indeed seems a naturally occurring exception to the rule that grafts exchanged between non-identical individuals will be rejected. Several theories have been proposed to explain this phenomenon [6, 69, 125]. Enhancement however might be the cause for the take of a foetus.

5. Summary

The acceptance of male skin grafts by the post partum femals with which they had been bred (Breyere and Barrett phenomenon) is mediated by enhancing antibodies as shown by passive transfer of antibodies, respectively by parabiosis and passive immunization.

Sensitization of multiparous females towards male antigens appears to occur, that probably in most cases results in the occurrence of enhancing antibodies.

CHAPTER VI B

FURTHER INVESTIGATION INTO THE MECHANISM OF ENHANCEMENT IN THE BREYERE AND BARRETT PHENOMENON

1. Introduction

There are two important alternatives to explain the mechanism of immunological enhancement. One centres about some sort of specific inactivation of the immune response in the lymphoid centres themselves, while the other depends upon a peripheral masking of the antigenic specificities of the transplant itself by bound antibody molecules. These two modes of action need not, of course, be mutually exclusive.

The following experiments were designed to reveal information about these two alternatives. The experimental model, described in the previous experiment, was used for the study of the mechanism. Enhanced skin grafts were transferred to normal recipients, isogenic to the mice, that carried the graft before (fig. 24).

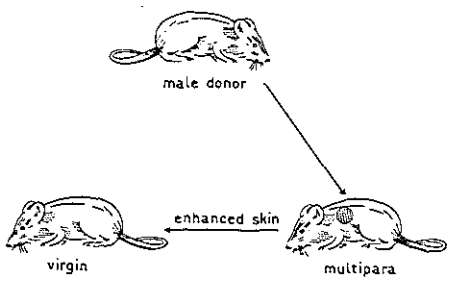


Fig. 24. Transfer of enhanced male skin from multiparous mouse to virgin.

2. Material and methods

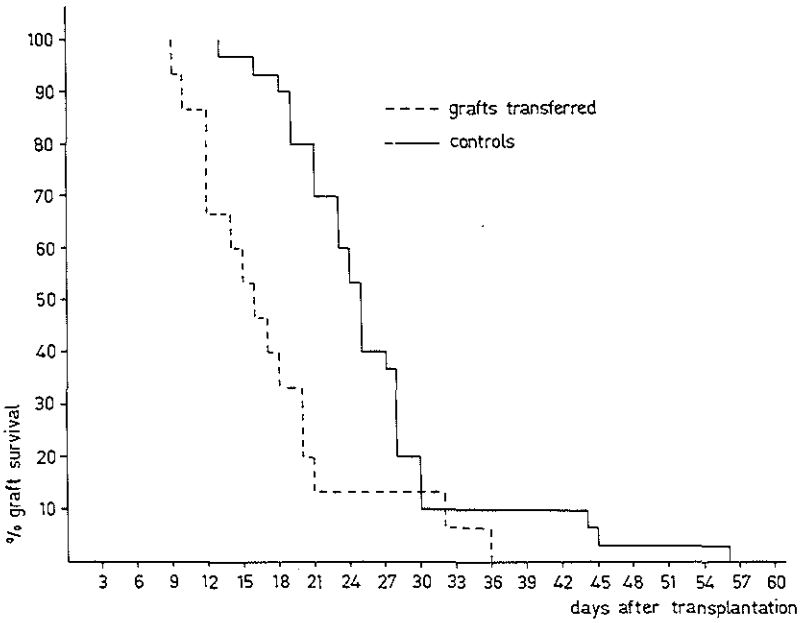
Normal adult $C_{57}BL/_6$, $C_{57}BL/_{10}$ and $C_{57}BL/_{10}$-LP mice were used throughout. In the $C_{57}BL/$strains the HY-locus exerts an obvious effect in that male to female grafts are predictably destroyed. Multiparous $C_{57}BL/_6$ females were healthy retired breeders acquired from the Jackson Memorial Laboratory in Bar Harbor, Maine.

3. Results

$C_{57}BL/_6$ male skin grafts were rejected with a MST of 26 ± 8.87 days (standard deviation of the mean) by adult virgin females, whereas their survival on

multiparous female recipients was extended to a MST of 35.9 ± 15.63 days. A graft was considered to be clearly enhanced if it was still 100 per cent viable at more than 10 days beyond the MST of the control group, since more than 90 per cent of control recipients had rejected their grafts by this time.

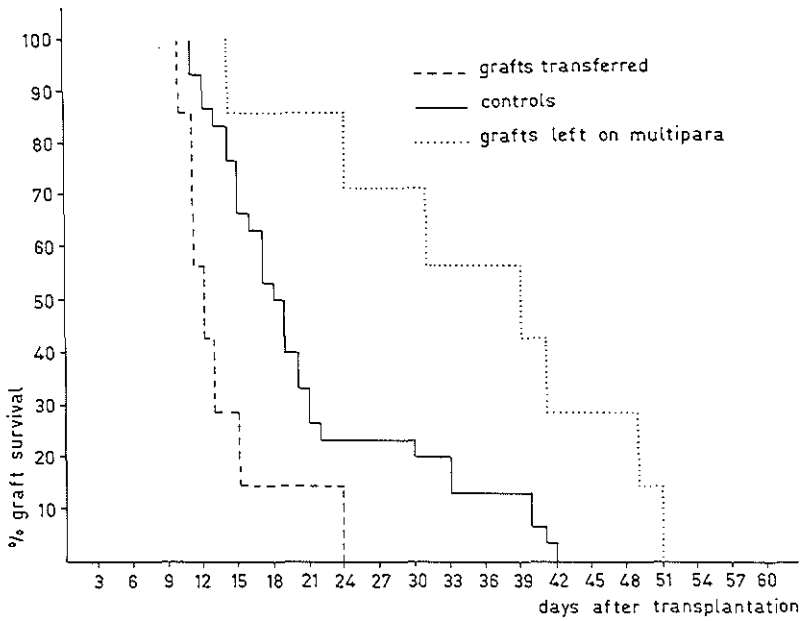
Secondary *transfer* of enhanced male skin grafts to adult females did not result in survival beyond that expected for normal male skin grafts. Indeed these grafts were rejected more rapidly than normal skin, as the MST of 15 secondarily transferred $C_{57}BL/_6$ male skin grafts was 17.6 ± 7.64 days rather than the 26,0 ± 8.87 days MST expected of normal skin (fig. 25). That the trauma of secondary transfer was not, in itself, an independent cause of this more rapid rejection was assured by the fact that enhanced male skin grafts survived indefinitely when placed upon isogeneic males.



		n	$\bar{x}$	s.d.	$P_{Student}$	$P_{Wilcoxon}$
————	controls	30	26.0	8.87		
- - - - -	transferred skin	15	17.6	7.64	0.001–0.002	0.0006

Fig. 25. ♂ skin transfer to virgin after enhancement in multipara.

The peculiar behavior of enhanced grafts on secondary transfer to normal recipients was emphasized by an experiment in which two skin grafts were simultaneously grafted from male $C_{57}BL/_{10}$ donors to multiparous $C_{57}BL/_{10}$ recipients. When both were shown to be enjoying enhanced survival, one graft from each recipient was transferred secondarily to an isogenic female. In all



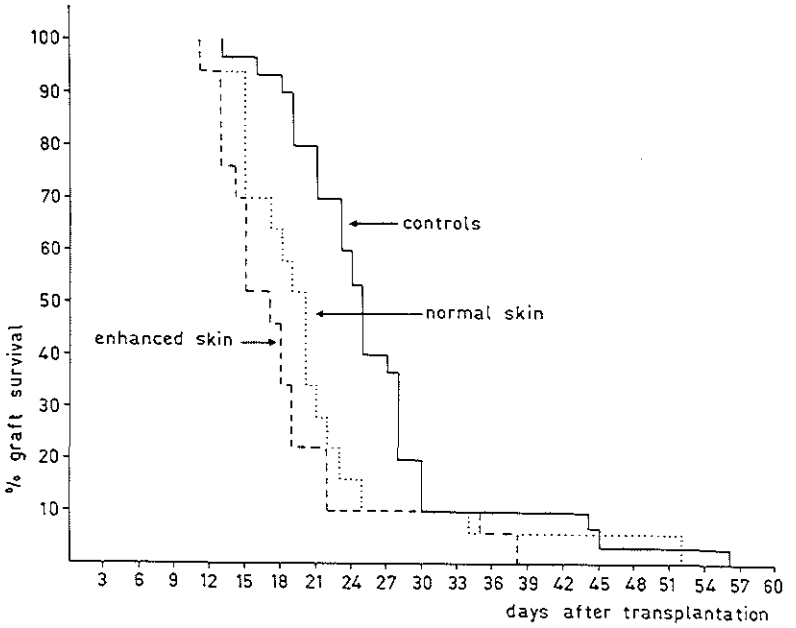
	male to virgine female	no. animals	graft survival (days)	standard deviation of mean
————	$C_{57}BL/_{10}$ controls male to female	30	21.2	9.58
– – – – –	transferred enhanced graft	6	13.5	5.24
.....	enhanced skin left on multipara	7	35.6	13.41

Fig. 26. Transfer of one of the two enhanced $C_{57}BL/_{10}$ male skin grafts.

instances the secondarily transferred graft was rejected significantly in advance of its partner which remained on the multiparous recipient (see fig. 26).

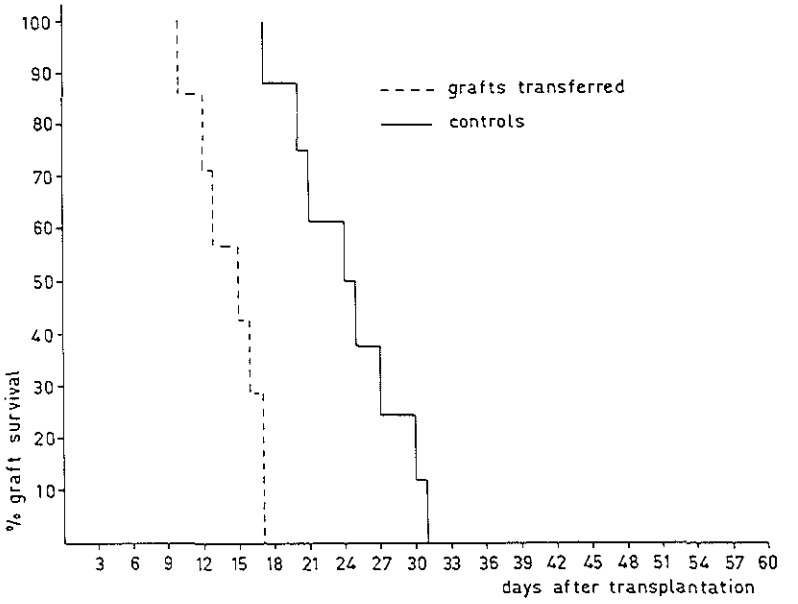
Using $C_{57}BL/_6$ mice in 17 instances male skin grafts were applied to normal females simultaneously with previously enhanced male skin grafts. In this experiment the previously enhanced grafts were rejected with an MST of 18.6 ± 7.30 days whilst the grafts from normal donors were rejected with an MST of 21.3 ± 9.44 days (see fig. 27). Both skin grafts were thus rejected at about the same time, a time significantly earlier than expected for normal skin. This again is an argument against a traumatic cause of the earlier rejection, for in that case one would not expect a more rapid rejection of normal grafts.

In an effort to shed some further light on this apparently non-specific effect, enhanced male $C_{57}BL/_{10}$ skin grafts were transferred instead to $C_{57}BL/_{10}LP$ male recipients. In this situation the recipient is presented not with the male-specific histocompatibility antigen(s) against which the enhancing antibody was directed but with the specificity determined by the H3-locus. Such grafts between normal individuals are rejected with an MST of 24 ± 4.9 days.



		n	$\bar{x}$	s.d.	P_{Student}	P_{Wilcoxon}
———	controls	30	26.0	8.87		
- - - - -	enhanced grafts	17	18.6	7.30	0.01–0.002	0.0005
·····	normal grafts	17	21.3	9.44	0.20–0.10	0.008

Fig. 27. Enhanced ♂ skin+normal ♂ skin on $C_{57}BL/_6$ virgin mice.



		n	$\bar{x}$	P
———	controls	8	24.4	
- - - - -	transferred skin	7	14.3	< 0.001

Fig. 28. Transfer of enhanced $C_{57}BL/_6$ ♂ skin to $B_{10}.LP$ ♂.

$C_{57}BL/_6$ male grafts, which have undergone enhancement on $C_{57}BL/_6$ females were, however, rejected much earlier by B_{10}.LP male recipients with an MST of 14.3 $\pm$ 2.69 days (see fig. 28).

4. Discussion

These observations do not seem to support the concept that immunological enhancement is a consequence of a peripheral effect, such as might occur if the histocompatibility antigens of the donor celles were masked by specific antibody. On the other hand, they suggest that enhanced skin grafts are peculiarly vulnerable to rejection when they are placed in an appropriate environment.

Indeed such enhanced skin grafts appear to have the capacity of provoking a heightened response on the part of normal individuals after secondary transfer. A potentiating effect between serum antibody and sensitized cells has previously been reported by others [14, 15] and such an effect may be operative with regard to the enhanced graft secondarily transferred to a normal recipient. A contribution toward this effect could also come from recipient leukocytes already mobilized into the base of the graft which are then carried with it onto the second host. Such sensitized "passenger" cells might contribute to the more rapid generation of a specific response in secondary recipients which would then cause an accelerated rejection of both the previously enhanced grafts and simultaneously placed normal grafts.

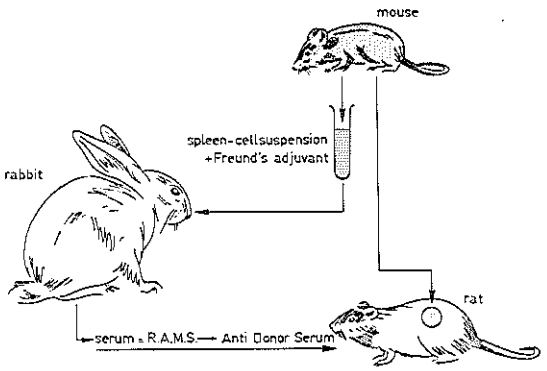
5. Summary

Enhanced male skin grafts appear to be particularly vulnerable to rejection after transfer to an isogenic nulliparous female. Thus enhancing antibodies do not seem to exert any protective effect on the graft itself.

The accelerated rejection of the enhanced grafts was even experienced to a lesser degree by simultaneously placed normal isogenic male skin grafts. The possible mechanism of this phenomenon is discussed.

CHAPTER VII

PASSIVE ENHANCEMENT OF SKIN HETEROGRAFTS IN RATS



1. Introduction

Heterografts and heterologous serum were explicitly excluded in the definition of enhancement, given by Snell [257]. Acceptance of heterologous grafts has been shown before with the use of ALS. Heterologous tumor grafts were also tolerated after pretreatment with lyophilized tissue early after birth [215]. However, the authors did not differentiate between tolerance and enhancement. Broder e.a. [41] reported the engendered growth of a mouse tumor in guinea pigs by incubation with Fab-fragments of isologous anti-tumor serum.

In the experiments described in this chapter an attempt was made to obtain enhancement of heterologous skin grafts. The results suggest that the extended survival of mouse skin grafts, observed in rats treated with antiserum, has been caused by immunological enhancement [130a].

2. Material and methods

Adult rats of the local inbred R strain and the inbred Wistar Wag/Ry strain weighing respectively 250–300 gm and 150–200 gm served as recipients for skin heterografts. Donors were $C_{57}BL/_6$ mice and guinea pigs. The experimental groups were injected intraperitoneally with 0.3–0.5 ml rabbit anti-mouse serum (R.A.M.S.), starting the day before transplantation. The serum-injections were continued for the first two days and subsequently every other day until day 17.

This antidonorserum (**A.D.S.**) was raised in rabbits by weekly injections in the footpads of $50–100 \times 10^6$ viable spleen- and lymphe node-cells from $C_{57}BL/_6$ mice with complete Freund's adjuvant. The rabbits were bled by cardiac puncture at different times after various immunizations. The skin grafts were transplanted following the technique of Billingham and Medawar [24]. Rejection was determined by macroscopical examination of the grafts. A chronic rejection pattern occurred in the rats, which were treated with antiserum. The reaction was characterized by progressive oedema and scab-formation. Serum and red cells were obtained by cardiac or retro-orbital sinus puncture from rats and mice respectively. For absorption studies 1 ml of the A.D.S. was incubated for 1 hour at 37 °C with 7×10^8 viable rat spleen cells.

Horse anti-mouse and anti-rat lymphocyte sera (H.A.M.L.S. and H.A.R.L.S. respectively) were kindly provided by the Radiobiological Institute, T.N.O. Rijswijk.

Cytotoxicity was determined by the microtiter method of Kissmeyer Nielsen as modified by van Rood [224]. Haemagglutinins were demonstrated by Stimpfling's method [268]. Both the Student Welch test and Wilcoxon test were applicated for evaluation of statistical significance.

3. Results

$C_{57}BL/_6$ skin grafts were rejected by R- and Wistar-rats in 6–8 days (M.S.T. 7 days, s.d. 0.61). Although the cytotoxic effect of normal rabbit serum on rat cells has been reported [66], no effect of normal rabbit serum was noted on the survival of mouse skin grafts in rats. Treatment of the recipients with *A.D.S.* (anti-donor serum), raised in rabbits, resulted in a striking prolongation of $C_{57}BL/_6$ skin graft survival up to 38 days (M.S.T. 19 days, $P_{\text{Student}} < 0.0001$, $P_{\text{Wilcoxon}} \leqslant 0.001$, see fig. 29+30).

A rather chronic rejection occurred in the experimental animals treated with A.D.S., in contrast to the rapid rejection of skin grafts by untreated controls. Another difference was the greater spread in survival times of the $C_{57}BL$ skin grafts in the treated rats. This type of rejection is characteristic for a weak histo-incompatibility.

It appeared that the A.D.S. should be prepared according to strict rules. Firstly, a long interval between collection and last injection of a hyper-immunization schedule is prerequisite for an effective serum. Significant prolongation of skin graft survival only occurred with those sera which were collected on the 15th day and later after the last immunization (see fig. 31). The effect of the A.D.S. seems to decrease, when obtained more than 49 days after the last immunization. Apparently, the formation of effective antibodies continues for a long time.

A relation seems also to exist between the number of immunizations and the

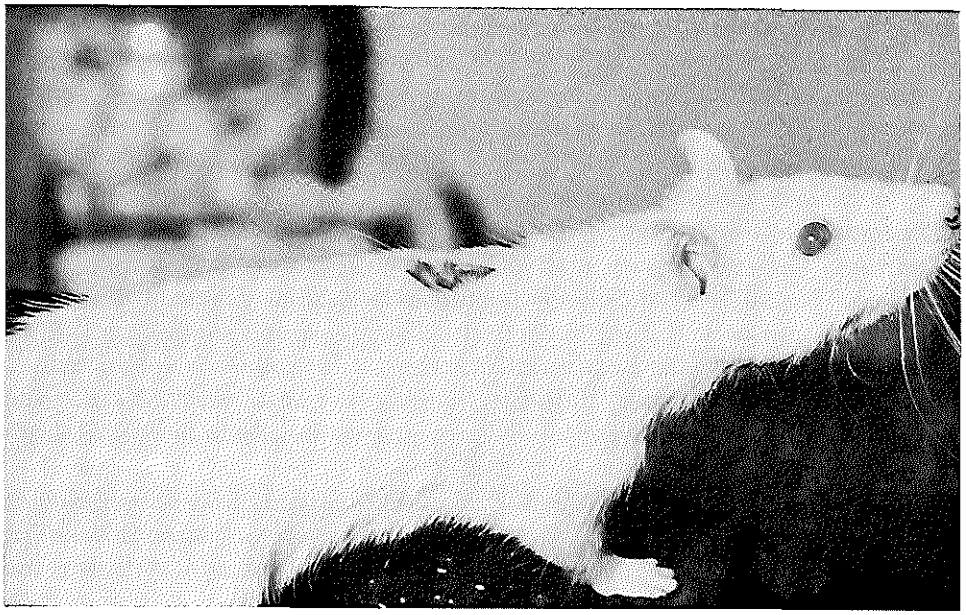
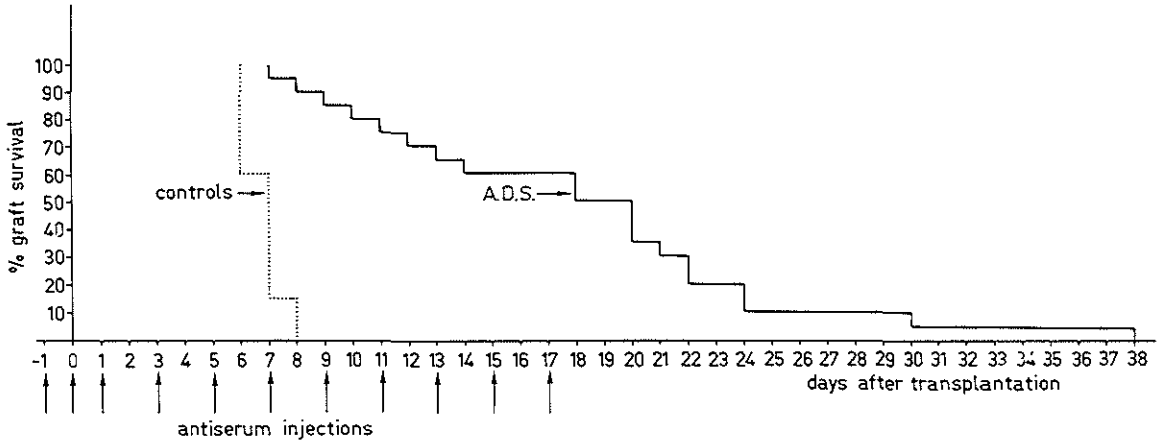
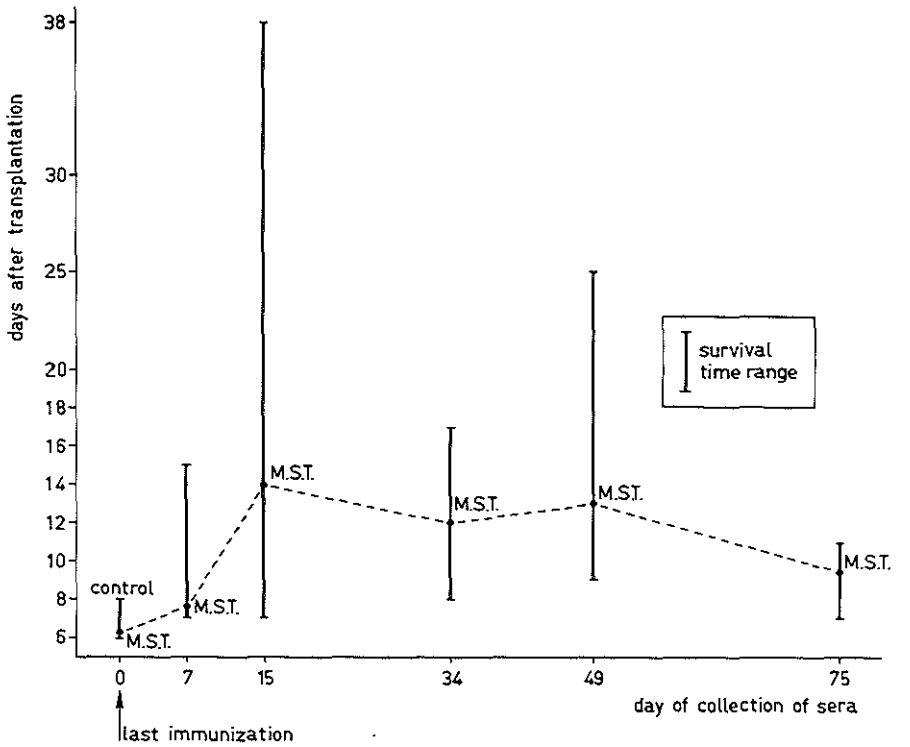


Fig. 29. Mouse skin graft on 32nd day after transplantation to rat.



Skin grafts mouse → rat 40 controls - - - - -
20 serum treated ——————

Fig. 30. Effect of anti donor serum on skin heterografts



Skin graft mouse → rat+serum collected on: 7 days after last injection
15 days after last injection
34 days after last injection
49 days after last injection
75 days after last injection

Fig. 31. Effect of sera collected on different days after the last immunization.

effectiveness of the sera (fig. 32). The most significant prolongation of graft survival was obtained with those sera that were collected after 14 weeks of immunization. These conditions also apply for sera to be used for the attainment of passive enhancement and antibody mediated humoral unresponsiveness and not for A.L.S. Various authors noted that enhancing sera could be best obtained long after the last injection of a hyperimmunization scheme, suggesting a change in antibodies after a certain time.

Secondly the addition of Freund's adjuvant to the cell suspension was found to be essential. In order to obtain a maximal extension of graft survival, the recipient should receive a sufficient number of serum injections. One injection on the day of transplantation or three injections on day minus one, zero and the day after transplantation did not influence the graft survival as significantly as did injections every other day after transplantation (fig. 33). Cytotoxic and haemagglutinating antibodies were present in very low titers in the rats tolerating the mouse skin grafts for a prolonged period. This might be an indication that central or afferent blockage of the immune response is the

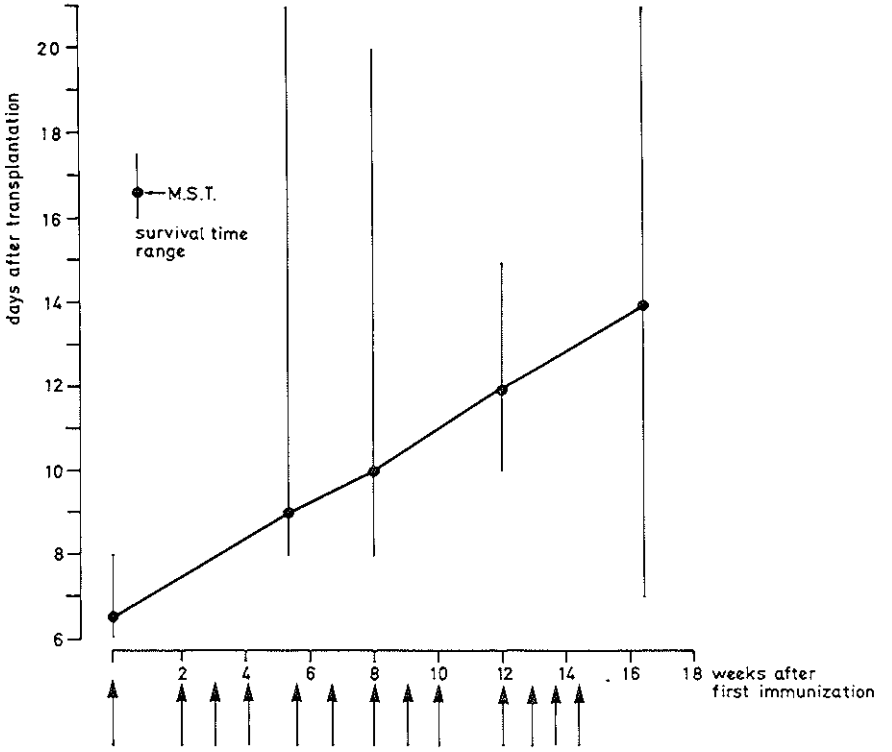


Fig. 32. Effect of various batches of A.D.S., collected after a different number of immunizations in the same rabbits, on the survival time of heterografts.

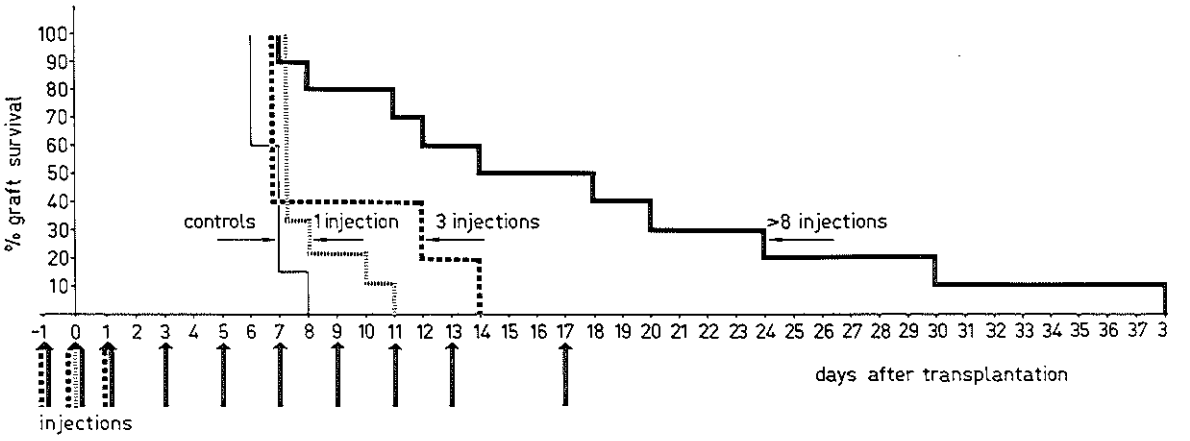


Fig. 33. Dose effects of A.D.S. on the survival of mouse skin grafts in rats.

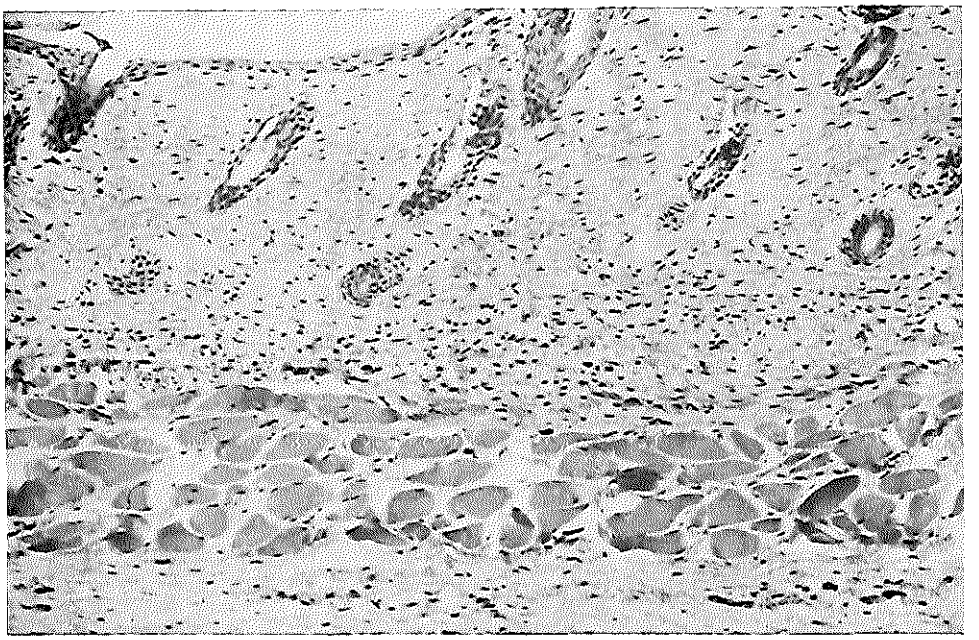


Fig. 34. Microscopy of mouse skin graft 28 days after transplantation to rats.

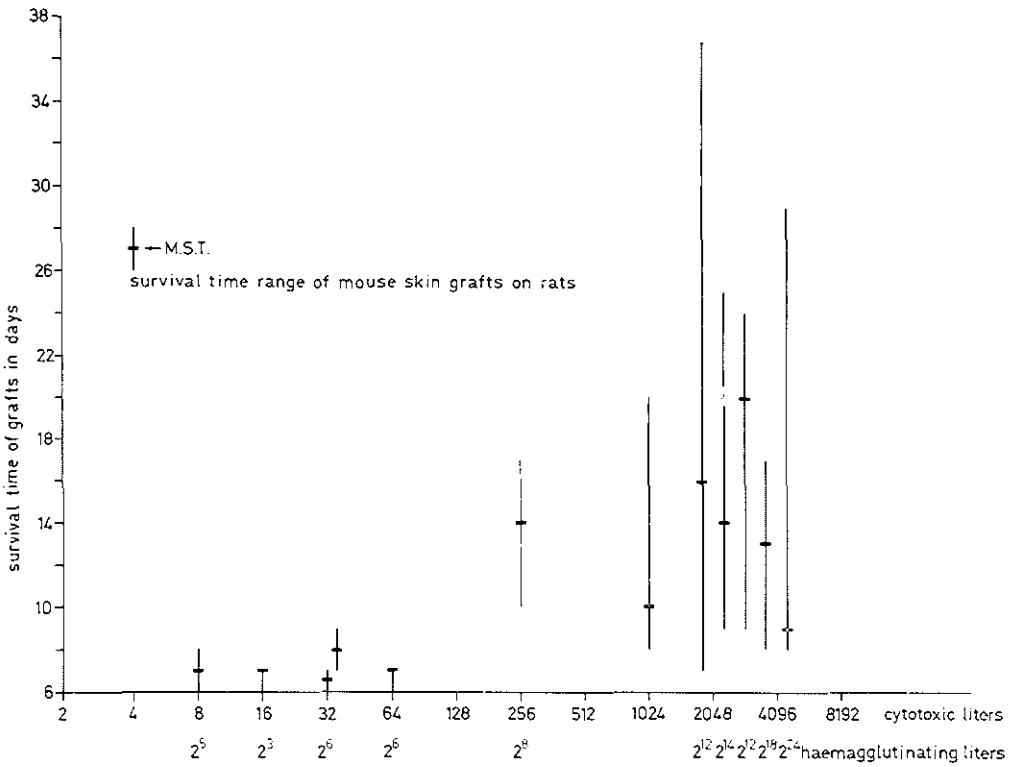
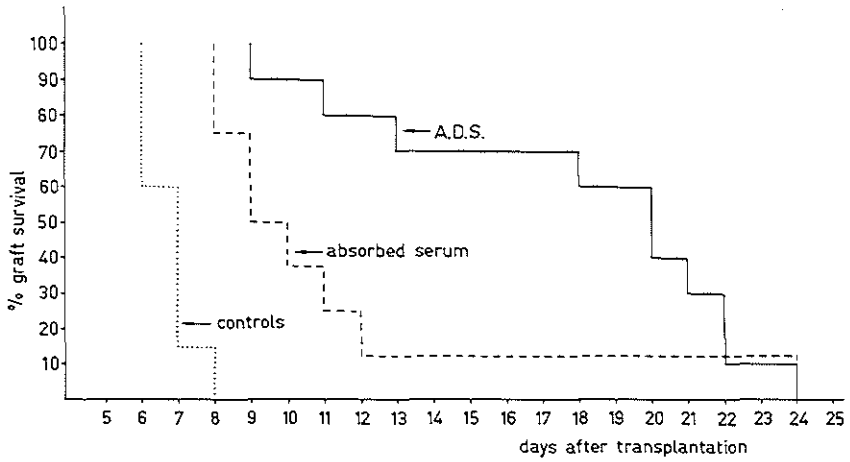
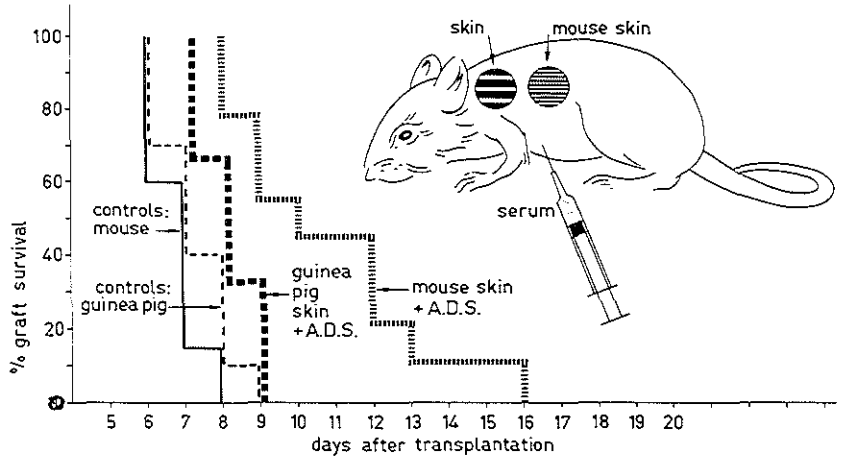


Fig. 35. Relation between cytotoxic- and haemagglutinating titers of passively transferred anti-donor serum and skin heterograft survival.



Skin grafts mous → rat
40 controls .....
10 + anti-donor serum ———
8 + absorbed serum - - - - -

Fig. 36. The effect of absorption of anti-mouse serum with rat cells.

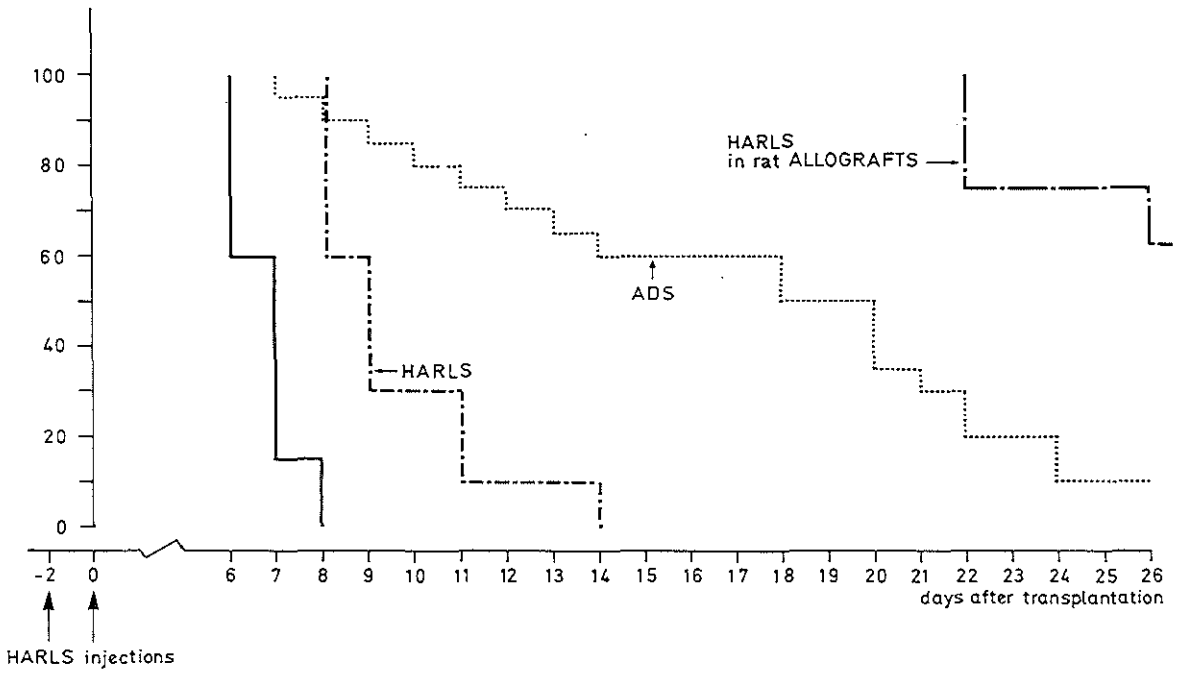


——— 40 controls mouse → rat
- - - - - 10 controls guinea pig → rat
9 guinea pig → rat + A.D.S.
9 mouse → rat + A.D.S.

Fig. 37. Effect of anti mouse serum on guinea pig skin grafts in rats.

cause of this phenomenon rather than efferent blockage. The abscence of signs of lymphocyte infiltrations and other immune reactivity in the surviving grafts, also seems to argue against efferent blockage (fig. 34).

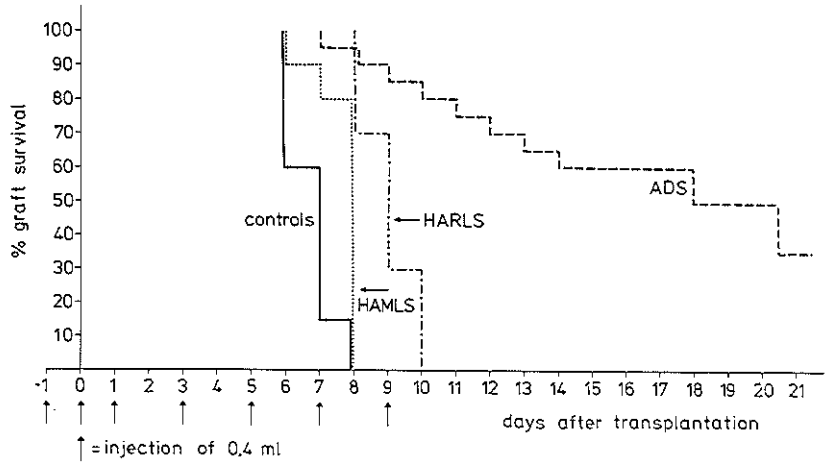
Many sera with high haemagglutinating and cytotoxic titers procured heterograft acceptance, whereas sera with titers lower than 256 were not effective (see fig. 35). For unknown reasons, some rabbits never produced enhancing sera, in spite of high titers. The question arises what mechanism might be responsible for the immunological unresponsiveness towards heterografts. Although the immunization schedules used seem to differ from those usually employed for the preparation of A.L.S., an A.L.S. effect should be more convincingly excluded. Indeed cross reaction of the R.A.M.S. with rat antigens existed. Antibodies against rat lymphoid and red blood cells were present, although to a lesser degree than the anti-mouse antibodies. To



Dose A.D.S.: *0.4 ml* every other day
Dose H.A.R.L.S.: *2 ml* on days −2; 0; +2

——— 40 mouse → rat controls
—·—·— 10 mouse → rat+H.A.R.L.S.
····· 20 mouse → rat+A.D.S.
.—.— 10 rat → rat+H.A.R.L.S.

Fig. 38. Effect of A.D.S. compared to A.L.S. (Horse anti rat lymphocyte serum or H.A.R.L.S.) on mouse skin grafts to rats.



———— 40 mouse → rat controls
. . . . . 10 mouse → rat + Horse anti mouse lymphocyte serum (H.A.M.L.S.) 0.4 ml/inj.
—·—·— 15 mouse → rat + Horse anti rat lymphocyte serum (H.A.R.L.S.) 0.4 ml/inj.
– – – – – 20 mouse → rat + A.D.S.

Fig. 39. Effect of A.L.S. compared to A.D.S. on skin heterografts. Dosage of both sera: *0.4 ml* every other day.

eliminate the anti-rat antibodies of the R.A.M.S., the serum was absorbed with 7×10^8 rat spleen and lymphe node cells per ml and subsequently injected into rats (see fig. 36). After absorption anti-rat titers reached much lower levels up to 1:16. Although the capacity of these sera to prolong skin graft survival was diminished, a significant effect on the heterografts remained.

The following experiment also seemed to exclude a simple A.L.S.-effect. In addition to a mouse skin graft, a guinea pig skin graft was applied to rats, which were treated with A.D.S. Assuming the existence of a recipient-specific A.L.S.-effect [13] one might expect an extended survival of both grafts. Immunological enhancement however is donor-specific and would only lead to acceptance of the mouse skin grafts. Indeed the guinea pig skin grafts were rejected in a normal period in contrast to the prolonged survival of the mouse skin grafts (see fig. 37). Yet a greater incompatibility probably exists between guinea pigs and rats. In that case the difference in graft survival of guinea pig and mouse skins could be partially explained by this discrepancy in histo-incompatibility.

One more argument against anti-lymphocyte antibodies being the active principle in A.D.S. was obtained by comparing a strong H.A.R.L.S. with our A.D.S. in the mouse skin to rat system. A.D.S. was much more effective than H.A.R.L.S. in prolonging heterograft survival, while the effect of H.A.R.L.S.

on allografts was among the best ever observed with A.L.S. preparations (fig. 38). Moreover, when H.A.R.L.S. was given in doses similar to those of the A.D.S., little effect on the heterografts was observed (fig. 39). Finally, an effective H.A.M.L.S., when employed as A.D.S., was not able to procure significant prolongation of heterograft survival.

4. Discussion

The results of these preliminary experiments indicate that at least in the mouse to rat combination, enhancement of heterografts is feasible. Enhancement of heterologous skin grafts has so far not been reported. A preferable method for the demonstration of enhancement is its passive transfer by serum.

In this experimental model *A.D.S.* produced a striking prolongation of mouse skin graft survival up to 38 days. Realizing the possibility of cross reactivity with rat cells, much effort was directed towards the exclusion of an A.L.S. effect of the A.D.S. Several findings were in contradiction with an A.L.S. activity. For the attainment of an effective A.D.S., a stringent immunization schedule must be followed, dissimilar to the A.L.S. immunization schedules usually employed. The necessity of collecting the serum late after hyperimmunization and of avoiding intravenous immunization and the indispensability of Freund's adjuvant are not typical requirements for the preparation of A.L.S. [70, 162, 247]. On the other hand, immunological enhancement has also mostly been induced with late hyperimmune sera. For humoral unresponsiveness too, the sera were collected at a time that antibodies occurred with a high affinity for antigenic determinants. Furthermore the activity of A.D.S. seems to be dependent upon high cytotoxic and/or haemagglutinating titers in contrast to A.L.S.. Sera with low titers were not effective.

The activity of A.D.S. could not be completely removed by absorption with rat cells. The latter finding indicates a specificity of the sera for donor antigens. Such a specificity for the antigens of the donor is characteristic for enhancement.

The difference between an A.L.S. and A.D.S. also became apparent in a different effect on heterograft survival. A much more pronounced acceptance of heterografts occurred with the use of A.D.S., whereas the H.A.R.L.S. (A.L.S.) produced very extended survivals of rat allografts. If A.D.S. acts as an A.L.S., it would be an unbelievably potent one. Moreover, no effect was obtained with H.A.R.L.S., when this A.L.S. was administered in the same doses as A.D.S. Neither did H.A.M.L.S. produce extended survivals.

All these results can be taken as evidence for enhancement as the cause of the prolonged survival of these heterografts. The absence of antibodies in the serum of rats displaying enhancement and the absence of histological reactions in the accepted grafts, suggest a central or afferent inhibition of the immune

response, because in those cases a cellular and humoral response are not elicited.

In conclusion enhancement may occur in a heterologous host-donor combination.

5. Summary

Prolongation of mouse skin grafts on rats by an anti-donorserum (*A.D.S.*) is demonstrated. Several findings suggest that enhancement causes this effect. The methods for the preparation of the A.D.S. differ considerably from the immunization schemes of A.L.S.

The effectiveness of an A.D.S. absorbed with rat cells and the normal rejection of a simultaneously placed guinea pig skin graft, were also not in favor of an A.L.S. effect. Furthermore, a much better prolongation of heterograft survivals was obtained with A.D.S. than with A.L.S. When administered in the same dosage as A.D.S., A.L.S. was not effective. The absence of antibodies and of a histological reaction in the accepted grafts, suggests a central or afferent inhibition.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

The results obtained from the present experiments shed some light on the multiple problems of enhancement. Since Casey reported in 1932 the „xyz"-effect or the enhanced tumor growth rate by "enhancing material" many investigations about this subject have followed, which were summarized in several reviews [33, 127, 139, 146, 150, 232, 257]. Immunological enhancement has been readily demonstrated with tumor allografts, whereas normal tissues were rather resistant to passive or active enhancement. Since the development of the technique of grafting vascularized organs in rodents, enhancement has been reported with vascular allografts [87, 98, 101, 167, 211, 212, 269, 270].

Yet, attempts to produce enhancement of skin allografts were relatively unsuccessful [22, 34, 60, 99, 119]. Either higher antigenicity of the epithelial tissue and a different vascular composition than tumor tissue and vascular organs, or a completely different type of rejection of skin grafts, as compared to vascularized grafts, might be responsible. In this respect the use of organ-specific antibodies might be important for the attainment of enhancement. The enhancing sera for tumors were usually prepared by injection of tumor tissue whereas the sera employed for the enhancement of skin graft were raised by lymphoid cell injections. The finding that rat skin tissue and bone marrow may share antigenic determinants that are not present on isologous lymphe node cells [242] could partly explain those results [30]. Obviously, it is of great importance to obtain enhancement of normal grafts. Eventual clinical application can only be based upon extensive experimental experience with enhancement of normal instead of tumor grafts. The outcome of the enhancement of skin grafts might be a useful indicator for the effectiveness of enhancement in the clinical situation.

1. **Enhancement of allografts** (chapters IV, V, VI A, VI B)

One of the most interesting aspects of our studies was that enhancement of skin grafts has consistently been obtained. *Active* as well as *passive* enhancement of skin grafts could be induced reproducibly in coisogenic strains of mice, i.e. strains that are genetically identical, except for a single specificity at the major histocompatibility locus. One might argue that such a small genetic disparity, as in this case, at the *31* specificitity of the H2-locus would facilitate any attempt to affect the rejection reaction. However, $B_{10}.D_2$ (31+) skin grafts

were rejected by B_6AF_1 (31—) controls in as short a time, as occurs across the strong H2-locus. Furthermore, the study of such weak transplantation antigens is of great value for clinical transplantation, because the improvements in tissue typing enable transplantation between matched individuals. At the present time the difference in histocompatibility antigens between donor and recipient can be carefully measured. For example, many well-matched donor-recipient combinations have been used in Eurotransplant [225]. Thus weak histo-incompatibilities frequently occur in clinical transplantation not only in related, but even in unrelated donor-recipient combinations.

The principal reason that *coisogenic* combinations were tested, was that attempts to produce enhancement across the strong H2-locus had so far been unsuccessful. It was also assumed that effective specific antisera could more readily be produced in combinations with a monospecific disparity. This was indeed the case, for active as well as passive enhancement have consistently been produced in this model.

Pretreatment of the B_6AF_1 recipients with either $B_{10}.D_2$ lymphoid cells (*active enhancement*) or B_6AF_1 anti-$B_{10}.D_2$ serum (*passive enhancement*), led to a significant prolongation of $B_{10}.D_2$ graft survivals. Passive enhancement was also demonstrated across the *32*-specificity of the H2-locus as well as across the 31- and 32-specificity together, which involved the enhancement of respectively $B_{10}.B_R$ and $(B_{10}.D_2 \times B_{10}.B_R)F_1$ skin grafts in pretreated B_6AF_1 recipients. Interestingly, a synergistic effect was noted between *irradiation* of the recipient and treatment with anti-31 serum.

Furthermore, enhancing antibodies also seem to circulate in multiparous mice, for it was demonstrated that the *Breyere and Barrett phenomenon* is based upon immunological enhancement. Enhancement of skin grafts occurred in multiparous females, using the male Y antigen as antigenic difference. In other words, the Breyere and Barrett phenomenon or the prolonged acceptance by postpartum females of tumor and skin grafts of the male strain with which they had been mated, is mediated by enhancing antibodies.

Thus, these experiments demonstrate that both active and passive enhancement of skin grafts can be consistently obtained in certain mouse models. The importance of this finding is, that in such simple models, the mechanism of enhancement and the structure of the enhancing antibodies can be studied.

2. **Enhancement of heterografts** (chapter VII)

Realising that the possibility to prolong graft survival is inversely related to the strength of the histo-incompatibility [121, 176, 177], a much stronger antigenic barrier was also tested. To this end, heterografts were studied instead of allografts. Between two species a stronger rejection reaction will be provoked than between members of the same species, for the magnitude of antigenic

difference is determined by the genetic disparity. Rats were chosen as recipients of mouse skin grafts, because of the evidence in the initial studies of Stuart for enhancement of vascularized grafts in this species. Chapter VII reveals that enhancement can be passively induced in a hetero-specific combination. Mouse skin grafts enjoyed a prolonged survival of up to 38 days in rats by the administration of late hyperimmune rabbit anti mouse serum.

3. Interpretation of the experimental data

Previously, heterografts had never been used in enhancement studies. Therefore, the concept of enhancement, which was restricted to the prolongation of allograft survival by allo-antiserum [257, 256], can now be extended to heterografts and heterologous serum. The effectiveness of enhancing sera in passive enhancement does not only seem to depend upon the genetic disparity, but also upon a specific production method of the sera.

Characteristic for an enhancing antiserum is the specificity towards donor antigens. It is an anti-donor serum and hence in these experiments denoted as *A.D.S.* It might even be important to use tissue-specific antigens for the preparation of A.D.S. intended for enhancement of a particular organ. Males have usually been employed preferentially for the induction of enhancement, because of their weaker immune response compared to females. In chapters IV and V, the difference in immune response even became apparent in a faster rejection by female mice of skin allografts incompatible at the 31- and 32-specificity of the H2-locus. Yet in these experiments no significant difference was noted in the degree of enhancement in females and males.

The physiological role of immunological enhancement is currently a matter of speculation. A regulatory function of antibodies by means of a feedback mechanism became apparent in the experiments with antibody mediated humoral unresponsiveness. This homeostatic mechanism may prevent excess antibody formation. Many different immunological homeostatic mechanisms probably exist in animals and man. The control mechanisms that govern the rate of cell division, or the occurrence of tumor cells, may be either humoral – by antibodies directed against new antigens on those cells – or intercellular by contact inhibition of cell proliferation [48]. Disturbance of one or more of these control mechanisms may give rise to the growth of tumors. Tumors, possessing specific antigens, might be protected from being rejected by enhancing antibodies. This mechanism has indeed been demonstrated by Hellström e.a. [112, 115] for instance in patients with either melanoma, coloncarcinoma or neuroblastoma. They developed an in vitro assay of lymphocyte-target cell interaction. Serum of tumor bearing patients contained factors that blocked the inhibiting effect of the lymphocytes of the patient on tumor colony forming. These blocking factors disappeared during regression of the tumor.

Homeostasis might also be interrupted by immunosuppression and it has been reported that a higher incidence of tumors occurs in patients undergoing immunosuppressive treatment.

Another activity of enhancement might take place in the remedy of bacterial diseases by preventing the occurrence of antigenic competition. Antigenic competition comprises the suppression of the immune response against a certain antigen by the simultaneous elicitation of a response to a second antigen [78]. Such an immune suppression could promote the multiplication of bacterial cells and in this way sustain infectious diseases. Enhancing antibodies might eliminate antigenic competition by blocking one of the antigens and thus enable a normal immune response to occur against the other antigen [217].

From the results, described in chapter VI, it appears that immunological enhancement is involved in the protection of the foetus. The foetal antigens are different from those of the mother and thus are an exception to the rule that grafts containing transplantation antigens will be rejected when placed in a genetically different surrounding. The various explanations for this phenomenon [6, 69, 125] have subsequently been contradicted. Pregnant females do seem immunologically competent. Controversy also exists about the anatomical barrier that prevents either a sensitization or rejection. There are supporters of an intrinsic antigenic deficiency of the trophoblast or its coating by an inert substance like mucoprotein. In contradiction to such a barrier is the evidence for feto-maternal transfer of lymphocytes and materno-fetal transfer of IgG [288].

Chapter VI reveals the existence of sensitization of multiparous females towards the male antigens and the occurrence of enhancement, proven by passive transfer of antibodies, by means of parabiosis and passive immunization respectively. Hellström [114] described an in vitro protective effect of a serum factor present in pregnant mice on foetal cells in the presence of isologous immune lymphe node cells. Thus immunological enhancement seems to occur in pregnant and multiparous mice. Enhancing antibodies may in some way protect the foetal graft and abortus may result from the abscence of those antibodies. It might be interesting to investigate why the enhancing antibodies are so readily formed in this graft-host relation and whether the sera of human multiparae also harbour enhancing antibodies.

4. Mechanism of enhancement

The mode of action of enhancing antibodies is still not understood. The occurrence of enhancement or rejection might depend upon the balance between enhancing antibodies and those antibodies which cause rejection in cooperation with the cellular reaction. In our experimental models passive

enhancement was used in preference, since the induction of enhancement by active immunization can be easily confused with the induction of tolerance. These experimental models enabled us to investigate the basic problems of enhancement.

Male skin grafts, that exhibited enhancement on multiparous mice, were transferred to isogenic normal females. The accelerated rejection of such grafts seems to argue against the concept of an efferent mechanism of enhancement. However, it does not completely exclude such a mechanism, because it remains possible that sensitized leucocytes and/or antibodies present in those transferred grafts, are no longer inhibited by the circulating enhancing antibodies. Although it is still generally accepted that antibodies alone do not play an important role in skin graft rejection, some investigators reported a destructive effect of antibodies on skin grafts [15, 61, 63, 64, 106, 107, 204, 262]. A dual effect of the same antiserum has also been reported [43]. Such an effect probably also plays a role in another experiment described in this thesis. Hyperimmune A.D.S., which induced immunological enhancement of skin allografts in mice, induced a slightly accelerated rejection of the skin allografts when injected once on the 7th day after transplantation.

Enhancing sera might thus be able to support and accelerate an already active rejection reaction. Whether "enhancing" antibodies support or inhibit immune reactions may well depend on the state of the host and the quality and distribution of various antibodies in the serum.

Two findings excluded an afferent blockage. The effectiveness of one injection of A.D.S. on the 4th day after transplantation in mice can not easily be explained by an afferent effect. This leaves us with two other explanations for the mechanism of enhancement, namely central and efferent inhibition of the immune response. The synergism of A.D.S. with simultaneously injected donor cells suggests that the immune apparatus is not affected centrally during enhancement.

In the case of an efferent blockage one would expect the immune reaction to occur in the presence of a live graft. Yet, in the rats with enhanced mouse skin grafts, antibody titers could not be demonstrated, although simple methods were used. A cellular infiltration was not present in the enhanced grafts. Furthermore, enhancement of a second skin allograft could be achieved in mice, provided that hyperimmune A.D.S. was injected during the first skin graft procedure. At the time that the second skin is grafted, passively transferred antibodies will not be left in the recipient in view of the short half-life time of mouse antibodies. Hence in this case the immune mechanism and memory probably have not been affected at the efferent level.

Investigations of antibody mediated humoral unresponsiveness, a phenomenon that might be closely related to enhancement, indicate the importance of antigen-antibody interaction. For the occurrence of this phenomenon, all an-

tigens have to be covered by antibodies. Likewise, following the theory of Möller, antibodies should be directed against all the antigenic determinants for the attainment of enhancement. Two observations were in contradiction with this theory. Firstly the occurrence of enhancement of mouse skin allografts by an A.D.S., which was only directed against one of the two existing antigenic determinants. Enhancement of ($B_{10}.D_2 \times B_{10}.B_R$) ($31^+$, 32^+) skin grafts occurred in B_6AF_1 (31^-, 32^-) mice by the administration of anti-$B_{10}.D_2$ serum. Secondly, an enhancing effect of anti-31 serum was even noted, when 5 other H2-specificities were present in the donor. The effectivity of a monospecific enhancing serum in a model where more specificities are involved, indicates that either not all the antigens of the graft have to be covered by enhancing antibodies or that cross reactivity exists. This finding is at variance with a peripheral blockage of enhancing antibodies.

In conclusion, these various experiments could not provide evidence for the existence of one single mechanism of immunological enhancement (table 18). Although the findings were mostly in favour of a central or afferent blockage, an efferent effect could not be excluded, especially in view of the impressive evidence for such a mechanism in the literature [99, 196]. It might be possible, that a combination of factors is operative in immunological enhancement. A protection of the graft at the efferent level may very well coincide with a central inhibition of the immune response.

Table 18. Summary of those experiments in this thesis that give information about the mechanism of immunological enhancement.

	mechanism of enhancement*		
experimental model	afferent	central	efferent
transfer of enhanced skin grafts (chapter VII B)	±	+	±
effectiveness of one injection on the 4th day after transplantation (chapter V)	−	+	+
synergism of A.D.S. and donor cells (chapter IV)	+	−	+
synergism of A.D.S. and irradiation (chapter V)	+	±	+
enhancement of second skin grafts (chapter V)	±	+	−
no antibody titer in enhanced rats no cellular infiltration in enhanced grafts (chapter (VII)	+	+	−

* +: probably the acting mechanism
−: probably not the acting mechanism
±: can not be excluded as the acting mechanism

5. Conclusion

The feasibility of the enhancement of skin allo-and heterografts as shown in the described experiments, opens the possibility for speculation about clinical

application. If these results can be repeated in other species and with vascularized organs, then immunological enhancement might provide a solution for some of the clinical transplantation problems. Enhancement could then also be applied to heterologous organ transplantation, which might be the future of clinical transplantation [222] because of the problems around allo-transplantation [261, 262]. The shortage of suitable organs imposes a restriction to allo-organ transplantation. Immunological enhancement could also be applied to human allografts, either by specific heterologous A.D.S., or homologous A.D.S. from multiparae. A.D.S. treatment could be combined with low doses of A.L.S. or other immunosuppressiva, for in the experimental model, described in chapter V (fig. 11), a synergistic effect of A.D.S. with irradiation and later with A.L.S. was noted [174].

Due to the present immunosuppressive treatment, many of the transplanted patients die of infection and some may develop tumors. Enhancement would probably avoid these complications. Although it is tempting to test enhancement in clinical transplantation, this should not be undertaken until enhancement is reproducibly attained in other species like dogs or monkeys and until more information is available about the mechanism and the exact structure of the antibodies, that are causing enhancement.

SUMMARY

The experiments described in this thesis concern the enhancement of skin allografts and heterografts in mice and rats respectively. Immunological enhancement of skin grafts could reproducibly be obtained in these experimental models. Before, enhancement had only been described for tumors and recently for vascularised grafts. The results of our experiments are described in the following chapters. All the data were statistically evaluated.

Chapter IV dealt with the effect of active immunization of the recipient with frozen-thawed or viable donor tissue. This resulted either in sensitization or *active enhancement*, depending on the route of administration, the number of injections and the time interval between the last injection and skin grafting. Even a second set response could be abolished in this experimental model.

In *chapter V* passive immunization of mice was studied. Antisera could be raised that, after passive transfer, consistently produced *passive enhancement* of mouse allografts. This model enabled further studies about the mechanism of enhancement. One of the findings was that passive enhancement of skin allografts could be obtained up to 4 days after transplantation. This excludes the possibility of an afferent mechanism.

The occurrence of active as well as passive enhancement appeared to depend upon a special hyperimmunization scheme. The same immunization method was successfully used for the attainment of good rabbit anti-mouse serum in the experiments concerning the enhancement of skin heterografts in rats. Besides such an artificial form of enhancement, induced by passive or active immunization, a naturally occurring form of enhancement appeared to exist in multiparous mice. Male skin grafts are known to be accepted for a longer period by isogenic multiparous females (*Breyere and Barrett phenomenon*). In *chapter VI A* it is demonstrated that this phenomenon is caused by an enhancing factor in the serum of multiparous mice, as proven by parabiosis of multiparous and nulliparous females and by passive transfer of this effect to nulliparous females. It is concluded that any pregnancy leads to a sensitization towards the foetal antigens. The effect of the sensitization is probably in most cases abolished by enhancing antibodies.

In *chapter VI B* the mechanism of enhancement in multiparous mice was studied by transferring enhanced skin grafts from multiparous mice to nulliparous mice. It appeared that the enhanced grafts were particularly vulnerable to rejection after the transfer. This makes a protective effect of the enhancing antibodies on the graft itself unlikely.

A synergistic effect was observed between passive enhancement and either irradiation of the recipient or treatment with donor cells. Although this finding suggests a peripheral mechanism of enhancement, other experimental

results denoted an inhibition of the immune response at the central or afferent level. For example, immune inhibition at the central level probably played a role in two experiments. One showed passive enhancement of a *second* mouse skin graft under appropriate conditions, the other an accelerated rejection of an enhanced skin graft after transfer from a multiparous to a nulliparous mouse. One single mechanism in the phenomenon of enhancement could not be demonstrated in spite of these and other data.

In *chapter VII* skin *heterografts* were tested in rats after the successful attempts to produce enhancement of skin allografts. Rats were chosen as recipients of the heterografts, because of the evidence for enhancement of vascular allografts in this species in the initial studies of Stuart [269]. It could be demonstrated that passive enhancement of mouse skin heterografts may be obtained in rats, although a strong histo-incompatibility exists in this combination.

One of the findings that may bring us closer to the study of enhancement in man is the effectiveness of *a heterologous* rabbit anti-mouse serum in rats as described in the last experiment [130]. Yet, because of the various still unknown factors, clinical application of enhancement should not yet be attempted.

SAMENVATTING

De experimenten, die in dit proefschrift beschreven zijn betreffen de enhancement van huid allografts en heterografts in respectievelijk muizen en ratten. Immunologische enhancement van huidtransplantaten kon in deze experimentele modellen bij herhaling verkregen worden. Voorheen was enhancement alleen aangetoond met tumoren en onlangs ook met gevasculariseerde transplantaten. De resultaten van de hier vermelde experimenten zijn in de volgende hoofdstukken beschreven. Alle gegevens werden statistisch geëvalueerd.

Hoofdstuk IV behandelt het effect van actieve immunisatie van de ontvanger met donorweefsel. Dit resulteerde hetzij in sensitisatie of *actieve enhancement*, afhankelijk van de wijze van toediening, het aantal injecties en het tijdsinterval tussen de laatste injectie en huidtransplantatie. Zelfs een second set response kon worden te niet gedaan in dit experimentele model.

In *hoofdstuk V* werd passieve immunisatie van muizen bestudeerd. Antisera konden vervaardigd worden, welke bij herhaling *passieve enhancement* van muizen allografts veroorzaakten. Dit experimentele model maakte verdere onderzoekingen van het mechanisme van enhancement mogelijk. Eén van de bevindingen was dat passieve enhancement van huid allografts nog tot 4 dagen na de transplantatie verkregen kon worden. Dit sluit de mogelijkheid van een afferent mechanisme uit.

Het voorkomen van actieve zowel als passieve enhancement bleek afhankelijk te zijn van een speciaal hyperimmunisatie schema. Dezelfde immunisatiemethode werd met succes toegepast bij het verkrijgen van een effectief konijnen anti-muizen serum in de experimenten betreffende enhancement van huid heterografts in ratten. Naast een dergelijke artificiële vorm van enhancement, veroorzaakt door passieve of actieve immunisatie, bleek er ook een natuurlijke vorm van enhancement te bestaan bij muizen. Het was reeds bekend dat mannelijke muizenhuid transplantaten voor langere tijd worden geaccepteerd door isogene multiparae dan door nulliparae (*Breyere en Barrett-fenomeen*). In *hoofdstuk VI A* wordt duidelijk gemaakt dat dit fenomeen wordt veroorzaakt door een bepaalde factor in het serum van de multiparae. Dit werd bewezen met parabiosis van een multipara en een nullipara en met passieve „transfer" van dit effect naar nulliparae. Uit de experimenten kwam naar voren dat elke zwangerschap tot sensitisatie voor foetale antigenen leidt. Het effect van de sensitisatie wordt waarschijnlijk in de meeste gevallen teniet gedaan door enhancing antistoffen.

In *hoofdstuk VI B* werd het mechanisme van enhancement bestudeerd in multiparae door „enhanced" muizenhuidtransplantaten over te plaatsen van multiparae naar nulliparae. De „enhanced" transplantaten bleken na het

overplaatsen versneld afgestoten te worden. Dit sluit een beschermend effect van de enhancing antistoffen op de transplantaten zelf uit.

Een synergistisch effect werd waargenomen tussen passieve enhancement en zowel bestraling van de ontvanger, als behandeling van de ontvanger met donorcellen. Hoewel deze bevinding een perifeer mechanisme van enhancement aangeeft, duidden andere resultaten meer op een centrale of afferente inhibitie van de immuunreactie. Een verhindering van de immuunreactie op centraal niveau speelde waarschijnlijk een rol in twee experimenten. Eén toonde aan dat passieve enhancement van een *tweede* muizenhuid onder bepaalde condities mogelijk bleek, het andere beschreef de versnelde afstoting van een „enhanced" huidtransplantaat na overplaatsing van een multipara naar een nullipara. Ondanks deze en andere bevindingen kon één enkel mechanisme van immunologisch enhancement niet aangetoond worden.

In *hoofdstuk VII* werden huid *heterografts* getest in ratten na het succes met de enhancement van huid allografts. Ratten werden gekozen als ontvangers van de heterografts, omdat Stuart onlangs in deze diersoort de enhancement van gevasculariseerde allografts had aangetoond. Hoewel een sterke histo-incompatibiliteit bestaat in de muis-rat combinatie kon passieve enhancement van de muizenhuid heterografts verkregen worden. Eén van de bevindingen, die ons dichter bij de bestudering van enhancement bij de mens brengt, is het goede effect van een *heteroloog* konijn anti-muizenserum in ratten, zoals beschreven in het laatste experiment [130]. Echter, gezien de nog vele onbegrepen factoren lijkt klinische toepassing van enhancement nog niet gerechtvaardigd.

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CURRICULUM VITAE

J. Jeekel werd geboren op 7 mei 1941 te Soerabaja. Na het afleggen van het eindexamen Gymnasium B te Breda in 1959 ging hij over tot de studie van de geneeskunde te Leiden, welke in 1966 met het artsexamen werd afgesloten.

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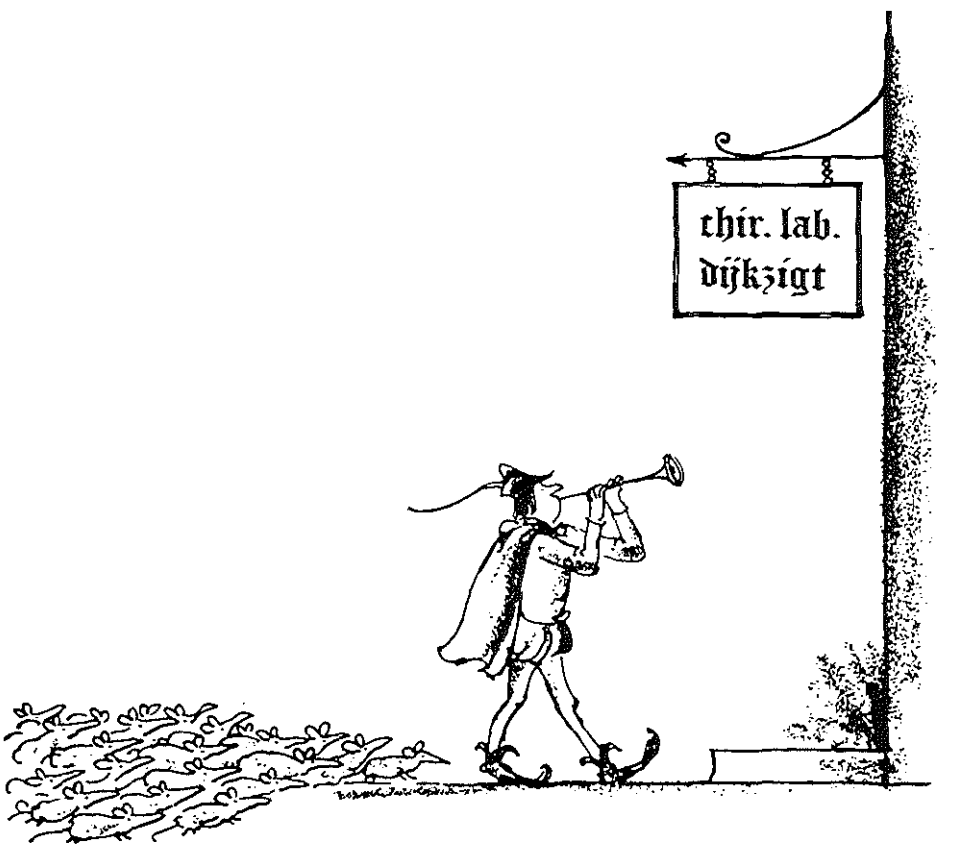
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