HYPERACUTE REJECTION AS A MODEL OF ANTIBODY-INDUCED VASCULITIS

M.J.J.T. Bogman

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Promotor: Prof. dr. R.A.P. Koene

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Aan mijn promotor.

CONTENTS

CHAPTER 1

INTRODUCTION

Hyperacute antibody-mediated graft rejection: Antibodies, Targets, and Mediators

The fate of transplanted tissues largely depends on the immune response of the recipient. This response is generated by antigens in the graft, termed transplantation antigens or histocompatibility antigens, that are genetically coded for by the histocompatibility genes. The most powerful inducers of immune defense mechanisms are located as a complex of closely related genes on one of the chromosomes, that is called the Major Histocompatibility Complex (MHC). The basic structure of the MHC of vertebrates of different species shows a remarkable homology. At present the best studied MHC regions are those of man, called the HLA-complex, located on chromosome 6, and those of mice, called the H-2 complex, which is located on chromosome 17.

The immune response of the host, generated by the histocompatibility antigens of the graft, evolves along two closely related main systems of defense: The cellular immune response, which leads to graft destruction by cytotoxic (T) cells, and the humoral response, in which graft destruction is effected by the action of specific antibody, directed against the graft tissue and produced by B cells. It has been shown that the antigens, coded by separate loci of the MHC, differ in the way in which they elicit the immune reaction of the host. In this respect two major groups of antigens have been distinguished: Class I antigens and Class II antigens. Class I antigens have a general tissue distribution and can be identified by their capacity to induce antibody formation in a non-identical host. These antigens are coded for by the HLA-A, B, and C loci of the HLA complex of man and by the H-2K and D loci of the H-2 complex of mice. They are also called SD (serologically defined) antigens. Class II antigens have a restricted cell distribution. They are mainly present on B cells, macrophages, monocytes, and activated T-cells, and are specifically involved in the induction of proliferative cellular immune responses. The MHC genes that code for the class II antigens are located on the HLA-D locus of the HLA

complex in man, and in the I region of the H-2 complex in the mouse. The Class II antigens of the mouse are also called Ia antigens, i.e. I-region associated antigens, those of man are also called DR antigens, i.e. D-region related antigens.

Early studies on graft rejection were mostly done with skin grafts in experimental animals, and they focussed on the cellular immune response of the host. The role of antibody, formed against the graft tissue was thought to be of minor importance (1,2). Later experiments, however, showed that antibodies could play an essential role in the rejection process. It was demonstrated that vascularized grafts could be hyperacutely rejected when antibodies against the graft tissue were circulating in the recipient, and that this type of acute graft rejection could be induced by passive transfer of serum from a sensitized animal into a non-sensitized recipient of the graft (3-5). The interest in the mechanisms involved in antibody-mediated graft destruction was further stimulated by the occurrence of hyperacute rejection of kidneys in clinical transplantation. In this dramatic situation the newly transplanted kidney shows blue-red discoloration within few minutes after the anastomosis has been made between the circulation of host and graft, and the kidney has to be removed immediately. Early clinical studies on this phenomenon revealed that it was strongly related to the presence of antibodies in the circulation of the host, directed against tissue antigens of the graft (6,7). Moreover, the finding that specific antibodies are also involved in chronic forms of rejection, thus accounting for a considerable number of graft losses, and that, contrary to the cellular immune response, the humoral response of the host is not very accessible to immunosuppressive therapy, has further increased the interest in the role of antibody in graft rejection.

The major part of our present knowledge of the factors involved in antibody mediated graft destruction is based on research in experimental animals. Of these the mouse is the most suitable species, since its MHC is the best known thus far. The availability of many inbred strains with accurately defined genetic backgrounds makes it possible to study immune responses across exactly defined MHC disparities. Studies of our group have been mainly performed in models in which mouse skin allografts or rat skin xenografts were transplanted onto the flanks of mice whose cellular immune response had been suppressed by treatment with anti-lymphocyte serum or Cyclosporin A. In these models acute antibody-mediated rejection (AAR) has been induced by i.v. injection of specific antibody directed against the graft antigens. Our studies on the mechanisms of antibody-mediated graft destruction have concentrated on three main topics: The biological activity of antibodies and fragments thereof,

factors determining the presence and the expression of the targets in the graft, and the role of mediator systems that act as effector mechanisms in the induction of the actual cell damage after binding of the antibody.

Part of the experiments, presented in this thesis, have been designed to gain a better insight in the mediation systems involved in the antibody mediated graft rejection. Since many of the conflicting theories in the literature on this subject have been based on histological observations, we thought it appropriate to use an approach in which not only the occurrence of macroscopic graft rejection but also the way in which the vasculitis histologically developed could be related to manipulations in mediator systems. We have therefore started with a description of the morphologic sequence of events in its relation to antibody dosage in AAR. Subsequently, we have studied the role of two mediator systems, viz. the complement system and polymorphonuclear granulocytes, that are thought to play an essential role in this process. In the last part of this thesis, we concentrate on the antigenic determinants of the endothelial cells, and especially on the question of whether Ia antigens can act as targets in the graft rejection, that takes place across disparities in the I region.

ARTHUS REACTION and SHWARTZMAN REACTION

Histologic patterns along which the vasculitis of AAR develops are often referred to as resembling an Arthus reaction or a generalized Shwartzman reaction (6,8-10). Thus, the terms Arthus-like and Shwartzman-like are used to define two characteristic and distinct patterns of vasculitis with essential differences in the sequence of events. In our studies on histologic features and mediator systems in AAR we also frequently use the terms Arthus-like and Shwartzman-like type of vasculitis, not only as definitions of the morphologic events, but also as a basis for discussions on differences in the pathogenesis between those types of reaction. Therefore, a short overview is given of the main characteristics both in morphology and in pathogenesis, of the two corresponding experimental models of vasculitis.

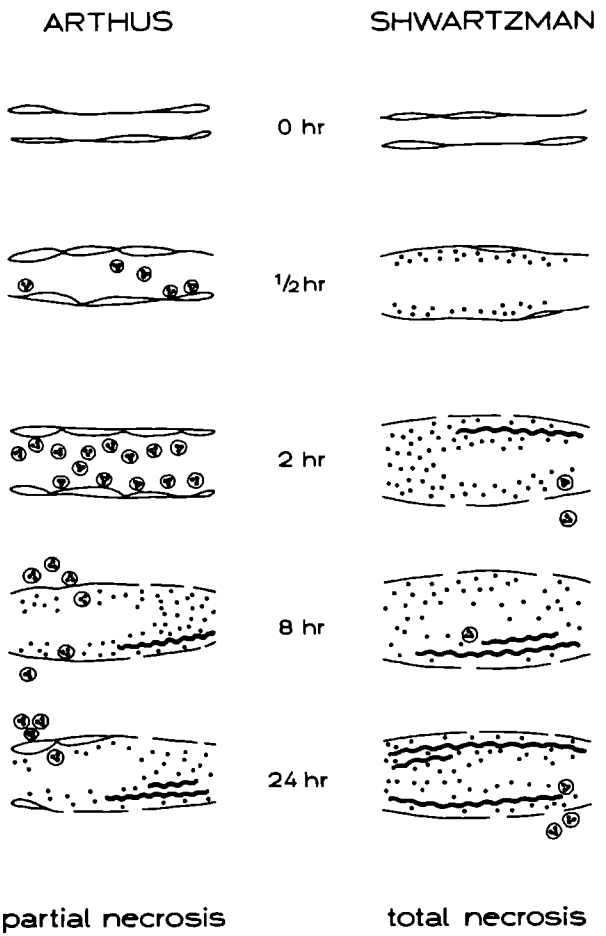

Fig. 1. Schematic illustration of the histologic sequence of events in the vessels in Arthus-like and Shwartzman-like types of vasculitis at 0, ½, 2, 8, and 24 hr after challenge.

Polymorphonuclear granulocytes.

Platelet aggregation.

Fibrin threads as visible in phosphotungstic acid-hematoxylin (PTAH) stain.

The Arthus reaction

The classic Arthus reaction is induced by intradermal injection of antigen in an animal that is immune to this antigen, i.e. in which antibodies against this antigen are circulating (11,12). The macroscopic reaction is a local swelling and redness of the skin, proceeding to intradermal bleeding and necrosis. In most instances, the maximal response is seen between 4 and 10 hr after the injection. Histologically, the reaction in the dermis is characterized by edema and marked accumulation of polymorphonuclear granulocytes (PMNs) in the vessels, visible from 30 min after the injection and maximal at 2-4 hr. Subsequently, the PMNs infiltrate the vessel walls and the perivascular tissue, and vascular damage occurs accompanied by platelet aggregation and intravascular coagulation. This is followed by further vessel wall destruction, bleeding, and, in severe cases, tissue necrosis. The morphologic sequence of events is schematically drawn in Fig. 1. Similar local reactions can be evoked when antibody is injected intravenously in a non-sensitized animal that is subsequently locally injected with antigen (direct passive Arthus reaction), by local injection of antibody in the skin and intravenous injection of antigen (reversed passive Arthus reaction), or by subsequent local injections of both antigen and antibody (local passive Arthus reaction) (12). The reaction is caused by the binding of antigen and antibody, resulting in precipitation of immune complexes against the vessel wall. The concomitant activation of the complement system leads to formation of chemotactic complement components of which especially C5a is a powerful attractant for PMNs. Neutrophils accumulate in the vessels and the eventual vascular damage is generally thought to be caused by the action of lysosomal enzymes released by the granulocytes during the process of phagocytosis of the immune complexes. Thus, the Arthus reaction is considered to be an immune complex vasculitis in which complement and PMNs are essential mediators in the induction of vessel wall damage (12). Prerequisites for the occurrence of the reaction are that the binding of antibody and antigen results in precipitation of immune complexes, that the antibodies are capable of complement fixation, and that complement and granulocytes are available. Elimination of complement from the circulation of the animal will prevent the reaction. Also, in the absence of neutrophils the reaction will not develop despite demonstrable precipitation of antigen, antibody, and complement in the vessels (12,13). It is generally accepted that platelets and the coagulation system play no essential role in the Arthus reaction, since several authors have reported that administration of heparin or depletion of platelets does not prevent the reaction (12,14,15).

The Shwartzman reaction

The classic generalized Shwartzman reaction is induced by an intravenous injection of bacterial endotoxins, administered within 24 hr after a first, so called preparing, i.v. injection of the same toxic agent (16,17). Following the second, or provoking, injection an acute generalized hemorrhagic necrosis occurs that is especially evident in the kidneys. In the local Shwartzman reaction the preparing injection is given intradermally, after which a second, intravenous injection provokes local hemorrhages in the skin (16,17). Microscopically the Shwartzman reaction is characterized by an acute necrotizing vasculitis, accompanied by early intravascular coagulation, that is not preceded by intravascular accumulation of PMNs (Fig. 1). The pathogenesis of this reaction is not clear. It has been reported that the destructive effect of the provoking injection can be abolished by anticoagulation or platelet depletion (18-20). The role of PMNs in the Shwartzman reaction has been a matter of debate (21,22) and the same holds for the role of complement (23,24). However, in contrast to the Arthus reaction, the Shwartzman reaction as such is thought not to be based on an immune reaction, and especially the local form of Shwartzman reaction has been shown to be non-specific. After a preparing local injection of endotoxin this reaction can also be elicited by an intravenous injection of other substances such as agar or glycogen (25). It is, however, not excluded that antigenic cross reactivity exists between these latter substances and endotoxin, which could explain the apparent absence of specificity.

It should be emphasized that both the Arthus and the Shwartzman reaction eventually result in vessel wall destruction and tissue necrosis, and that the differences in the histologic sequence of events are only clearly apparent in the most early stages (Fig. 1).

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CHAPTER 2

PATTERNS OF VASCULAR DAMAGE IN THE ANTIBODY-MEDIATED REJECTION OF SKIN XENOGRAFTS IN THE MOUSE

M. José J.T. Bogman, Jo H.M. Berden, Jacqueline F.H.M. Hagemann, Cathy N. Maass, and Robert A.P. Koene

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Patterns of Vascular Damage in the Antibody-Mediated Rejection of Skin Xenografts in the Mouse

M. José J. Th. Bogman, MD, Jo H. M. Berden, MD, Jacqueline F. H. M. Hagemann, Cathy N. Maass, and Robert A. P. Koene, MD

Established rat skin grafts carried by immunosuppressed mice were acutely destroyed by an intravenous administration of mouse antirat lymphocyte serum. The histologic pattern of destruction was dependent on the amount of antiserum administered. At low doses (0.01 ml) an Arthus-like reaction was seen with early accumulation of granulocytes. At high doses (0.25 ml) a Shwartzman-like pattern occurred, with early intravascular thrombosis and without evident participation of granulocytes in the initial phases. Groups of mice that received intermediate doses showed graft changes that were transitional between these two types of destruction. Similar histologic patterns have been described in clinical transplantation. Our results show that they are not fundamentally different and that the severity of the triggering reaction determines which of either type will occur. (Am J Pathol 1980, 100:727–738)

IN THE FIRST STUDIES of hyperacute rejection of human kidney grafts in patients with preexisting humoral antibodies against the major histocompatibility antigens of the donor it was found that the renal vessels showed extensive intravascular coagulation, suggesting the presence of a Shwartzman-like reaction.[1–3]

In a subsequent report Myburgh et al [4] showed that hyperacute rejection could also occur with histologic changes only consisting of intravascular neutrophil accumulation without fibrin deposition. In these Arthus-like reactions cortical necrosis with fibrin thrombi in the vessels may eventually occur, but coagulation is not seen in the initial stages as it is in the Shwartzman-like reaction. The relationship between these two types of antibody-mediated graft destruction and the factors that determine the occurrence of either reaction have not been clarified.

In an experimental model of hyperacute rejection in the mouse, in which established skin allografts carried by immunosuppressed animals were acutely destroyed by a single intravenous injection of antiserum, together with heterologous complement, we found a dense accumulation of polymorphonuclear granulocytes in the graft vessels, without deposition of fibrin.[5] This histologic picture is suggestive of an Arthus reaction and

From the Departments of Pathology and Medicine, Division of Nephrology, University of Nijmegen, Sint Radboudziekenhuis, Nijmegen, the Netherlands.

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Address reprint requests to M J Bogman, Department of Pathology, Sint Radboudziekenhuis, Geert Grooteplein Zuid 24, 6500 HB Nijmegen, the Netherlands.

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was very similar to the description in the reports of Myburgh et al.[4] In a comparable model of greater antigenic disparity, in which established rat skin grafted to immunosuppressed mice was destroyed by the administration of mouse antirat serum, the microscopic appearance was also compatible with an Arthus reaction. However, at later stages the grafts in this model showed extensive intravascular coagulation.[6]

So far, Shwartzman-like reactions have not been described in models of antibody-mediated skin graft destruction. We assumed that the histologic differences of graft destruction in the allogeneic and xenogeneic combinations were related to the antigenic differences between donor and host in the two models. A xenograft presents the host with a higher amount of foreign antigens than an allograft. It is conceivable that, in a xenogeneic combination, the administration of antibodies will lead to a higher concentration of antigen–antibody complexes in the graft, resulting in a greater generation of secondary destructive mediators, such as complement and chemotactive factors, and thus leading to a more violent histologic reaction.

We have now used another approach to vary the concentration of antigen–antibody complexes by administering different doses of destructive antiserum in the same donor–host combination. In this report we demonstrate that the patterns of vascular damage that can occur in the acute antibody-mediated rejection of xenografts in the mouse are directly related to the amount of destructive antibody administered. Low amounts of antibody gave rise to an Arthus reaction, whereas high amounts caused early-occurring intravascular coagulation, resembling a Shwartzman reaction. Intermediate amounts of antibody showed changes that were of a transitional type.

Materials and Methods

Animals

Inbred C57BL10 mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine) and inbred PVG/c rats from the Institute of Psychiatry, Bethlem Royal Hospital, Beckingham (Kent, U.K.). The strains were kept in our laboratory by continuous brother–sister matings.

Antiserums

Rabbit antimouse lymphocyte serum (RAMLS) was prepared according to the method of Gray et al.[7] Mouse antirat lymphocyte serum (MARS) was prepared in C57BL10 mice by weekly intraperitoneal injections of 5×10^7 PVG/c lymphoid cells suspended in complete Freund's adjuvant. After five injections the animals developed ascites, which was tapped weekly. The lymphocytotoxic titer of the ascites fluid was the same as the titer in the serum of these mice. The antiserums were pooled and sterilized by passage through a sterile 20-mμ filter (Schleicher and Schüll, Dassel, West Germany). IgG fractions (RAMLG and MARG) of both antiserums were prepared by ammonium sulfate precipitation and af-

ter dialysis purified by column chromatography on Sepharose 4B-CL coupled protein A The eluates were dialyzed, neutralized, and concentrated to their original cytotoxic titer Lymphocytotoxicity of the antiserums was assessed in a trypan blue exclusion assay [8] The titer of MARG was 1 20,480

Transplantation Procedure

Male PVG/c tail skin was grafted onto the flank of male C57BL10 recipients by a modified fitted graft technique [8] The fate of the graft was followed by daily macroscopic examination Grafts were considered to be rejected when no viable epithelium remained Median survival times (MST) and standard deviations (SD) were calculated according to the method of Litchfield [9] To postpone cellular rejection of the grafts, we treated all recipients with RAMLG on Days −2, −1, 0, 2, and 4 after grafting in a dose of 0 25 ml intraperitoneally This immunosuppressive regimen prolonged the median survival time (MST) of PVG/c grafts to 23 0 ± 1 2 days

Acute Rejection Studies

On Day 7 after grafting, when all grafts were well healed and showed no signs of rejection, the recipients received MARG via an intravenous injection in the tail vein Macroscopic aspects and histologic features were studied in four groups of mice that had received 0 01, 0 05, 0 1, and 0 25 ml of MARG, respectively, on Day 7 after transplantation.

Histologic Examination of the Grafts

The skin grafts and surrounding recipient skin were removed at different times after the intravenous injection of MARG and fixed in 4% buffered formalin From paraffin-embedded tissue blocks 4-μ sections were cut, and they were stained with hematoxylin and eosin (H&E), with elastic van Gieson stain for visualization of the vessel walls, with Azan stain for accentuation of the graft borders, and with Mallory's phosphotungstic acid–hematoxylin (PTAH) stain to detect fibrin The pathologist (M J B) who studied the histologic sections had no prior knowledge of the experimental protocol used The presence of fibrin deposits in the Mallory PTAH stain was taken as evidence for the presence of intravascular coagulation Platelet aggregation was assumed to be present when homogeneous concentrations of platelets were seen in a circular distribution along the vessel walls or obliterating the vascular lumen Polymorphonuclear granulocyte (PMN) accumulation was defined as the presence of polymorphonuclear granulocytes in the vessels in numbers exceeding that of erythrocytes and not in evident correlation with platelet aggregation

Results

MARG in a dose of 0.1 ml invariably caused macroscopically visible, acute graft destruction within 24 to 48 hours after injection, as was shown in a preliminary experiment in which 19 recipients of rat skin grafts received this dose of antiserum. We then administered the destructive antiserum in varying doses to four experimental groups of mice. The effects on graft survival as estimated by macroscopic inspection are given in Table 1. With few exceptions the histologic data were based on observations in groups of three mice each. The main histologic findings in four groups of similarly treated mice are schematically summarized in Text-figure 1. Details of the histologic examinations are given below.

In the group that received the lowest dose of antibodies (0.01 ml) the

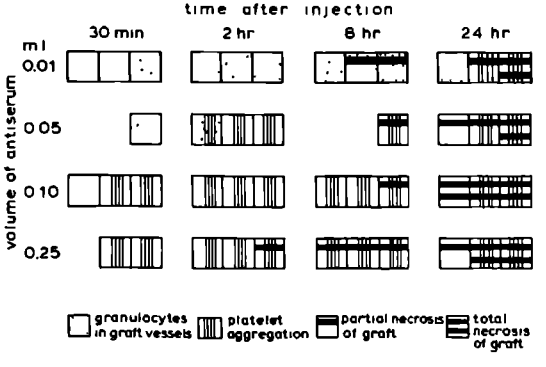
Table 1—Destruction of Rat Skin Grafts by Mouse Antirat Lymphocyte IgG

Volume of antiserum administered (ml)	Number of animals	Day of rejection* after transplantation
0.01	2	23, 30
0 05	2	11, 30
0.10	2	8, 9
0.25	3	8, 8, 9
—†	15	23.0 ± 1.2‡

* Grafts were considered to be rejected when no viable epithelium remained.
† Control animals.
‡ Median survival time (MST).

first reaction consisted of an increase of granulocytes in the graft vessels, first present at 30 minutes after the injection and maximal at 2 hours (Figure 1A and B). This incipient granulocyte accumulation was not accompanied by platelet aggregation or intravascular coagulation. Edema and vasodilatation were not predominant, and there was no congestion. From 2 hours after the injection of antiserum there was a progressive accumulation and extravasation of granulocytes (Figure 1B and C). Extravasation of erythrocytes was first evident at 2 hours and severe at 8 hours. It was always accompanied by destruction of the endothelium of the vessels. At 8 hours some of the grafts showed incipient or partial necrosis (Figure 1C). Only at 24 hours after injection were there signs of intravascular coagulation, as evidenced by platelet aggregation and the presence of fibrin in the PTAH stain (Figure 1D). However, these signs were limited to the necrotic areas. Outside these areas no intravascular coagulation or platelet aggregation was seen at any stage after the injection of the antiserum in this group.

The grafts that were followed macroscopically showed local signs of ne-



TEXT-FIGURE 1—Schematic illustration of the histologic findings in the skin grafts after the injection of increasing volumes of antiserum (MARG). Each square represents the observations in a single mouse. One mouse in the group that received 0.10 ml antiserum showed no reaction 30 minutes after injection.

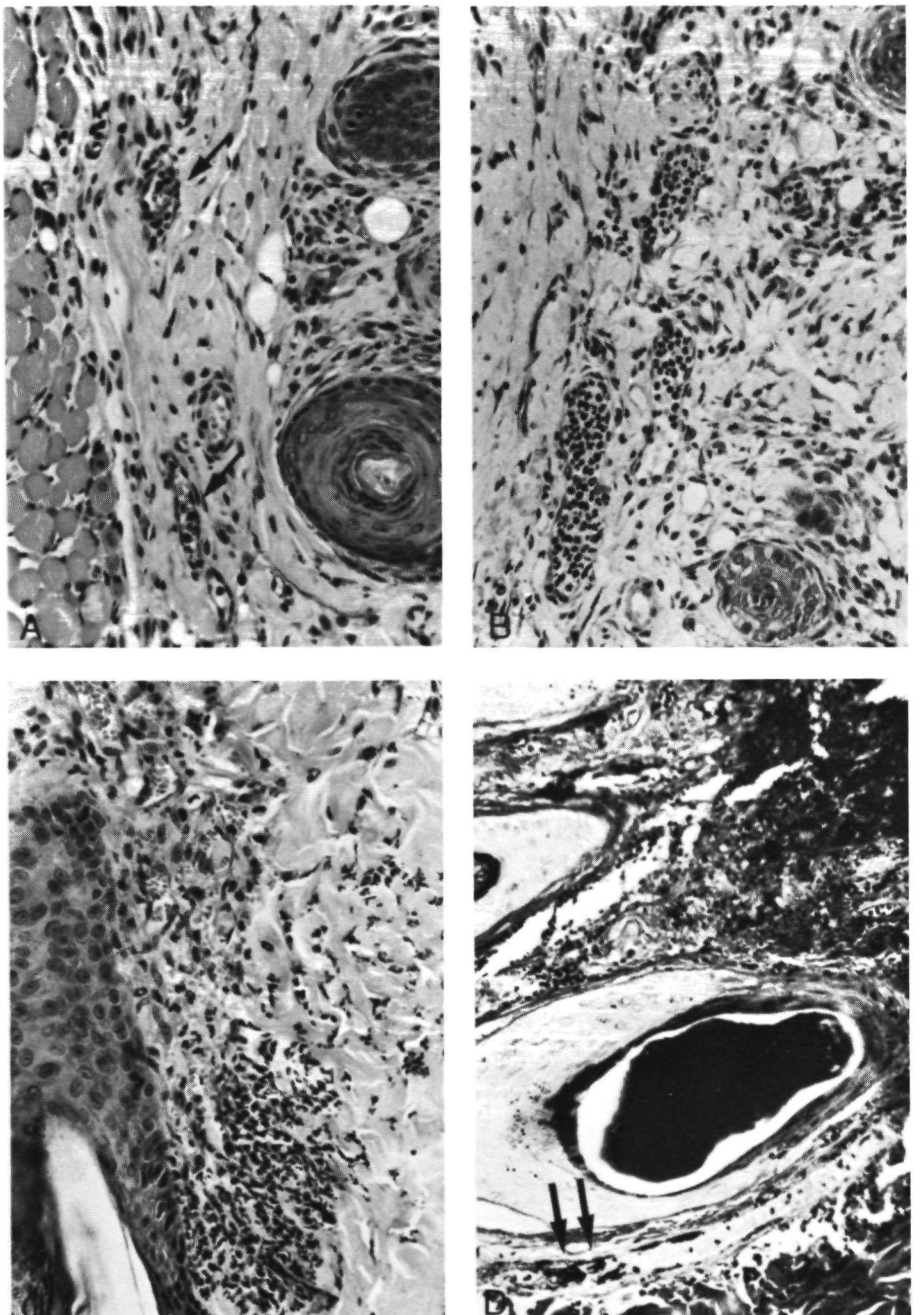


Figure 1—Histologic changes after intravenous injection of 0.01 ml MARG. The epidermal surface of the graft is oriented to the right. **A**—30 minutes: increase of polymorphonuclear granulocytes in the vessels (*arrows*). (H&E, ×78) **B**—2 hours: accumulation of granulocytes in the vessels is maximal. (H&E, ×78) **C**—8 hours: marked extravasation of granulocytes. Partial destruction of endothelium and extravasation of erythrocytes. Local necrosis in the graft. No intravascular coagulation is seen. (H&E, ×78) **D**—24 hours: local necrosis of the graft with intravascular coagulation and platelet-fibrin thrombi only in the area of necrosis (*arrows*). (PTAH, ×78)

crosis, which seemed to be reversible. Apparently, the amount of antiserum given was not sufficient to cause complete rejection of the grafts, since ultimate destruction occurred between Day 23 and Day 30, which is in the range of the graft survival in control mice.

In the group of mice that received the highest dose of MARG (0.25 ml) the first reaction was an aggregation of platelets along the vessel walls of the graft, which occurred as soon as 30 minutes after the injection (Figure 2A). This aggregation was accompanied by endothelial swelling and incipient destruction, vasodilatation, hyperemia, and extravasation of erythrocytes. These changes had increased 2 hours after injection (Figure 2B), and at this point local necrosis was already present with an increase of granulocytes in the recipient vessels underneath the graft. No accumulation of PMNs was seen in the graft vessels themselves and only a slight increase in those vessels in which platelet aggregation was present. At 8 hours there was clear-cut and total to subtotal endothelial destruction, and from this point fibrin threads were visible within the vessels (Figure 2C). At this stage scattered granulocytes were found in the vessels only in those areas where platelet aggregation or intravascular coagulation was also present. Microscopically the grafts showed total to subtotal necrosis after 24 hours (Figure 2D). Table 1 shows that, with this dose of antibody, macroscopic rejection was completed 24–48 hours after the injection.

The group of mice that received 0.10 ml of MARG showed a reaction pattern that was in broad outline similar to that of the group that received the highest dose, with the exceptions that in this group platelet aggregation at 30 minutes was somewhat less and that the first necrosis was seen not after 2 hours but after 8 hours.

Otherwise the histologic picture was identical.

In the group of mice that received 0.05 ml of antiserum one of the grafts showed, at 30 minutes after injection, a slight increase of PMNs in the graft vessels. At 2 hours 1 of 3 mice showed PMN accumulation in the vessels in one part of the graft and platelet aggregation in another. The two other mice of this group showed at this moment platelet aggregation without conspicuous PMN accumulation. Scattered PMNs were only seen in relation to platelet aggregation and were considered to be reactive to this aggregation. At 8 hours after injection considerable platelet aggregation could be observed, together with the presence of fibrin in the vessels and the occurrence of partial necrosis. At 24 hours these phenomena were more severe, 1 of the mice showing complete necrosis of the graft. From the 2 mice that were followed macroscopically in this group 1 showed hyperacute rejection of the graft at Day 11, while the other graft was not affected by the MARG injection (Table 1).

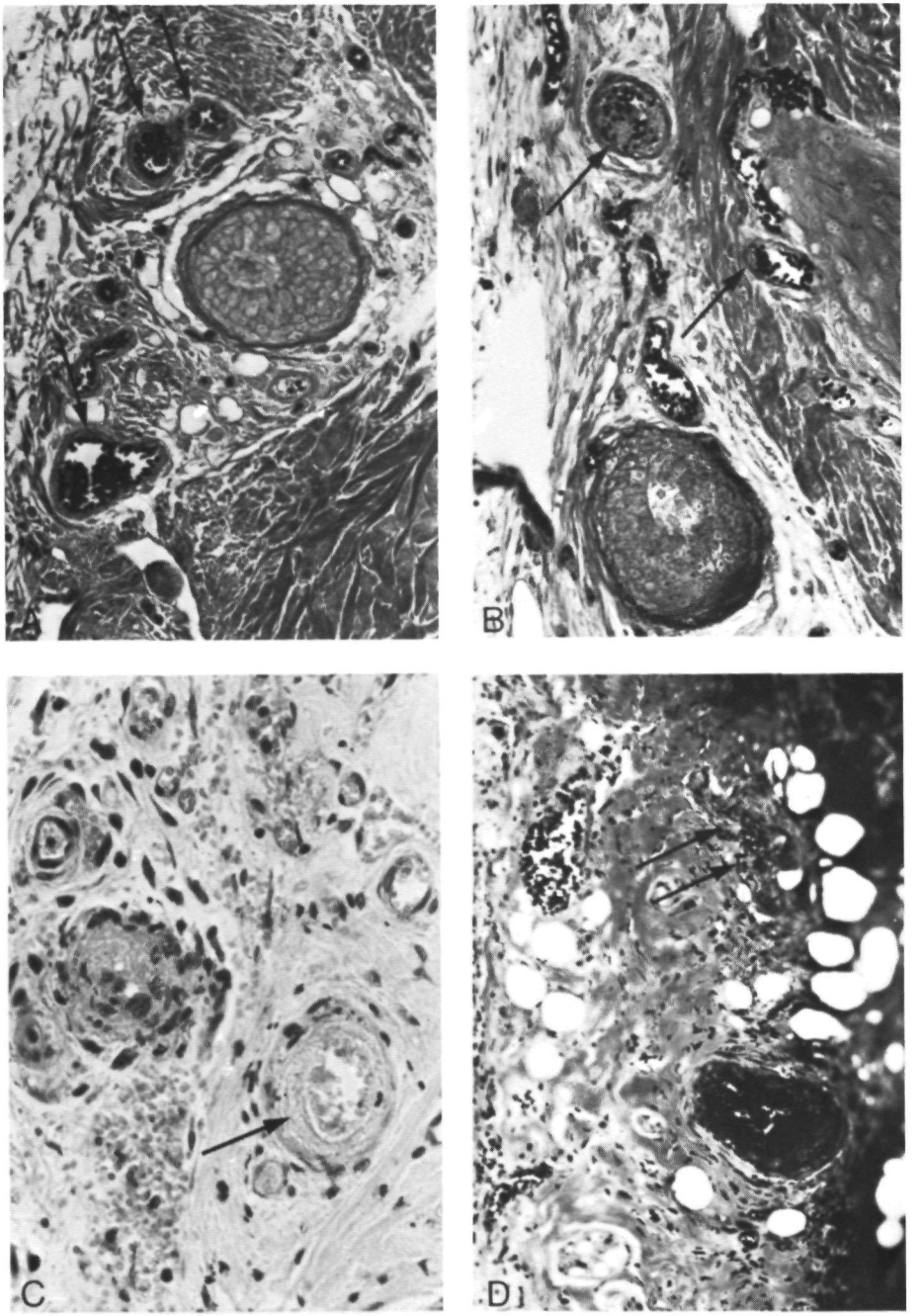


Figure 2—Histologic changes after intravenous injection of 0.25 ml MARG. **A**—30 minutes: incipient accumulation of thrombocytes along the vessel walls and vasodilatation (*arrows*). Erythrocytes are black; platelets are gray. (Azan, ×78) **B**—2 hours: accumulation of thrombocytes has increased; thrombi are partly occluding the vascular lumens (*arrows*). (Azan, ×78) **C**—8 hours: accumulation of thrombocytes along the vessel wall with deposition of fibrillar material (*arrow*). Granulocytes are not present in increased numbers. (H&E, ×125) **D**—24 hours: subtotal necrosis of the graft with destruction of vessel walls. Obstruction of the graft vessels by platelet fibrin thrombi (*arrows*). (PTAH, ×78)

Discussion

Our results confirm the clinical observations [3,4] that in acute, antibody-mediated graft rejection both Arthus-like and Shwartzman-like reactions can be present. They shed some light on the factors that determine which type of histologic pattern will occur. A relationship between both types of reaction is strongly suggested by our finding that it is possible to induce a transition from one type of reaction into the other by simply increasing the amount of destructive antibody. The differences between the reactions were most clearly seen in the histologic sections of the early phases of graft rejection. At the terminal stages, when necrosis of the graft had occurred, the reactions became almost indistinguishable. This finding is in accordance with previous reports, that, in considering the similarities and differences of the classic Arthus and Shwartzman reactions, study of the early phases is essential to evaluate the results.[10,11] Our model, in which low doses of antiserum were administered, can be considered as a direct passive variant of the Arthus reaction. The antigens against which the destructive antibodies are directed are present at the graft site, and most likely the histocompatibility antigens on the endothelial cell membrane are the primary target for the antibody. Binding of the antibody sets going a chain of events that via complement activation and PMN attraction leads to endothelial damage [10,12] and, if the reaction is severe enough, to intravascular coagulation. Morphologically the early accumulation of PMNs is the most salient feature. The findings in the group of mice that received the lowest dose show that such an accumulation does not necessarily lead to coagulation and necrosis. Some grafts escaped from destruction or showed only patchy necrotic changes.

In the two groups of mice that received the highest doses of antiserum (0.10 and 0.25 ml) there was acute thrombosis in the graft vessels without an increase of PMNs in the early phases of the reaction. A mild increase of PMNs in the vessels did occur during the process of thrombosis, but before this was seen there was already an abundant accumulation of platelets. It is likely that the PMNs were attracted secondarily by chemotactic factors, released during the clotting process.[13] In the generalized Shwartzman reaction, which is classically provoked by two separate injections of endotoxins, there is also an acute intravascular coagulation in which PMNs are absent from the thrombi [14] or present only in very few numbers.[15] Although these alterations closely resemble the histologic pattern found in our experiments with the high doses of antiserum, the mechanism of tissue damage is probably not the same. The generalized Shwartzman reaction is probably initiated by a direct activation of the coagulation process.[15] The

thrombi seem to consist mainly of fibrin without many platelets.[15,17] The hyperacute rejection in the mice receiving the high doses of antibody is clearly initiated by endothelial damage with consequent platelet aggregation and thrombosis (Figure 2A-C). This reaction is also different from the so-called local Shwartzman reaction, in which the preparative dose of the inciting agent is given intradermally.[14] In this reaction PMNs are abundantly present in the tissue before the second, provoking injection is given.[16] The presence of PMNs seems to be even essential here, since granulocyte depletion with nitrogen mustard will prevent the reaction.[16] Furthermore, in the local Shwartzman reaction PMNs form a conspicuous part of the thrombi.[14,15,17] Whether PMNs are necessary for the generalized Shwartzman reaction is a matter of debate.[15,18,19]

Because of these differences between the antibody-mediated graft rejection and the classic Shwartzman reaction, it seems a simplification to classify them in the same category. It is therefore more approriate to describe the graft rejection in terms of the mechanism involved and to try to explain the different patterns on this basis. The following description seems to accommodate both types of reaction, and the hypothesis on which it is based can be tested experimentally.

Small amounts of antibody fixed to antigen on the vessel wall will cause only little endothelial damage. Consequent complement activation with generation of chemotactic factors will attract PMNs.[20] These will phagocytose immune complexes [21] and release injurious constituents,[22] which will cause further damage to the endothelium.[17,21-23] The damaged cells in their turn attract more PMNs [24] and thus further enhancement of the reaction can occur. As soon as destruction of endothelium and disruption of the basement membrane or lamina elastica interna of the vessels occur, exposure of the thrombogenic [25,26] subendothelial tissue takes place. When larger amounts of antibodies are injected, endothelial cells may become completely destroyed by a direct cytolytic action of antibody and complement, implying that the thrombogenic subendothelial tissues will become immediately exposed to the blood flow, without intervention of PMNs, and rapid thrombosis will already have begun before chemotactic factors can become operative. Since the two reactions are based on the same inciting mechanism, one may expect that they can occur together in the same graft. This is exactly what we found in the grafts of animals that received intermediate doses of antiserum.

This interpretation of our results implies that in the group that received the high dose procedures to prevent coagulation or platelet accumulation will make the grafts accessible to PMNs and will thus change the Shwartzman-like pattern into an Arthus-like pattern. On the other hand, severe

depletion of PMNs will not prevent graft destruction, provided the dose of antiserum is so high that it can cause immediate intravascular coagulation. There are indications that this latter phenomenon indeed can occur. Cochrane has reported that in experiments designed to prevent a classic Arthus reaction by PMN depletion, the amount of antibody administered should not exceed certain levels. Otherwise a severe intravascular coagulation can occur.[12]

Our experimental model of graft destruction by different doses of antiserum provides a relatively simple tool with which one can study the involvement of the different factors mentioned. It can thus contribute to a better understanding of the mechanisms of vascular damage in the rejection process.

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CHAPTER 3

THE ROLE OF COMPLEMENT IN THE INDUCTION OF ACUTE ANTIBODY-MEDIATED VASCULITIS OF RAT SKIN GRAFTS IN THE MOUSE

M. José J.T. Bogman, Jo H.M. Berden, Ine M.H.A. Cornelissen, Cathy N. Maass, and Robert A.P. Koene

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The Role of Complement in the Induction of Acute Antibody-Mediated Vasculitis of Rat Skin Grafts in the Mouse

M. JOSÉ J. T. BOGMAN, MD,
JO H. M. BERDEN, MD,
INE M. H. A. CORNELISSEN, CATHY N. MAASS,
and ROBERT A. P. KOENE, MD

From the Departments of Pathology and Medicine, Division of Nephrology, University of Nijmegen, Sint Radboudziekenhuis, Nijmegen, The Netherlands

Rat skin grafts carried by immunosuppressed mice can be acutely destroyed by intravenous administration of mouse anti-rat antibody. The velocity of the reaction and the histologic sequence of events depend on the amount of antibody administered: low doses give an Arthus-like rejection, whereas at high doses a Shwartzman-like pattern occurs. Depletion of C3 by cobra venom factor treatment did not prevent acute rejection after intravenous injection of high doses of antiserum but changed the reaction from a Shwartzman-like to an Arthus-like pattern. Conversely, supplementary administration of rabbit complement caused a violent Shwartzman-like graft destruction after injection of low doses of antibody, which in complement-normal mice gave an Arthus-like reaction. The results show that complement can greatly amplify the antibody-mediated immune vasculitis and can substantially modify its histologic pattern. It is, however, not an absolute requirement for the occurrence of the destructive process. (Am J Pathol 1982, 109:97–106)

THE IMPORTANT ROLE of complement as an amplification system in acute antibody-mediated graft rejection (AAR) has been repeatedly demonstrated, both in clinical kidney transplantation[1] and in experimental allografts[2–5] and xenografts.[6,7] In two experimental systems, in which AAR was induced by the administration of specific antibodies to immunosuppressed mice carrying skin allografts or xenografts, the participation of complement in the rejection process was extensively studied.[4,6,7] In the allograft system the weakly acting endogenous complement of the mouse appeared to be insufficient to provoke hyperacute rejection after the administration of high doses of alloantibody specifically directed against the graft antigens. Concomitant administration of heterologous complement from rabbit, guinea pig, or human origin was necessary to induce acute graft destruction.[2] However, if mice were grafted with rat skin, which obviously represents a larger antigenic difference, the endogenous complement system proved to be sufficient to induce acute destruction after the administration of anti-rat antibody. In this model it has been shown that AAR could be induced in C5-deficient mice but less easily in mice treated with cobra venom factor (COVF), suggesting that especially activation of C3 was necessary for the amplification of the inflammatory reaction.[6] These findings suggested that complement fixation was essential for hyperacute rejection to occur, but a recent study has raised doubts about the general validity of this assumption. Experiments of Berden et al[7] showed that acute antibody-mediated destruction of skin xenografts can be induced both in C3-depleted and in C5-deficient mice, or, alternatively, with non-complement-fixing antibody, provided that the dose of antibody is sufficiently raised.

Insight into the role of complement, its mode of action, and the subsequent histologic events is not

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Address reprint requests to M. J. Bogman, Department of Pathology, Sint Radboudziekenhuis, Geert Grooteplein Zuid 24, 6500 HB Nijmegen, The Netherlands.

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only important for our understanding of the mechanisms that lead to antibody-mediated graft rejection, but may also give information about closely related processes based on immunologic injury of blood vessels, such as serum-sickness, autoimmune or allergic vasculitis, and glomerulonephritis. Together with similarities in pathogenesis, these processes share histologic features that have been compared with those of experimental Arthus and Shwartzman reactions.[8-10] The morphologic resemblance of acute antibody-mediated rejection to Arthus and Shwartzman reactions has been described both in human kidney allograft rejection[11-14] and in experimental allografts[2 15 16] and xenografts.[6 17] We showed in a previous report that acute antibody-mediated rejection of rat skin xenografts in mice can develop either along an Arthus-like pattern, characterized by early accumulation of polymorphonuclear granulocytes (PMNs) in the graft vessels, subsequent intravascular coagulation, and eventual necrosis, or along a Shwartzman-like pattern characterized by early intravascular coagulation.[17] Which of these patterns would develop was solely dependent upon the amount of antibody injected, high doses giving the more violent Shwartzman-like rejection, low doses causing a more slowly developing Arthus-like reaction, which nevertheless also led to total necrosis of the graft within 72 hours in about 50% of the animals. We assumed that the intensity of the reaction in the graft vessels was directly related to the number of antigen-antibody complexes in the graft. If the amount of fixed antibody is sufficient to cause immediate and complete destruction of the vascular endothelium, the subsequent exposure of the subendothelial tissues will cause early intravascular coagulation, consistent with a Schwartzman-like reaction. If the amount of antibody only causes partial endothelial damage, the ultimate necrosis will be brought about by destructive mediators, ie, PMNs, attracted by chemotactic factors, thus generating an Arthus-like pattern of rejection.

If our conclusion is right that the histologic pattern along which the acute antibody-mediated graft rejection develops is directly related to the destructive power of the first attack of antibody on the endothelial cells, it is to be expected that variations in the availability of complement, as one of the main mediators of the damaging process, can cause the same differences in reaction patterns to certain doses of antibody. In this report we demonstrate that C3 depletion by COVF treatment of immunosuppressed mice, carrying established rat skin grafts, could not prevent the acute rejection induced by intravenous administration of high doses of antibody but changed its pattern from a Shwartzman-like reaction to an Arthus-like one. Conversely, the supplementary administration of rabbit complement, together with the injection of low doses of antibody, could considerably enhance the effect of these low doses and change an Arthus-like pattern of acute rejection into a Schwartzman-like one.

Materials and Methods

Animals

Inbred strains of C57Bl/10 mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine) and inbred PVG/c rats from the Institute of Psychiatry, Bethlem Royal Hospital, Beckingham (Kent, U.K.). In our laboratory these strains were kept by continuous brother-sister matings. Goats, used to prepare anti-lymphocyte serum, were bought on the local market. The serum of New Zealand white rabbits was used as a source of complement.

Antiserums

Mouse anti-rat lymphocyte serum (MARS) was prepared in C57Bl/10 mice by weekly intraperitoneal injections of 5×10^7 PVG/c lymphoid cells suspended in complete Freund's adjuvant (CFA). After five injections the animals developed ascites, tapped weekly. The lymphocytotoxic titer of the ascites fluid, as determined in a trypan blue exclusion assay, was the same as the titer in the serum of these mice. We pooled and heated the antiserums at 56 C for 45 minutes to destroy complement activity, and sterilized them by passage through a sterile 20-mμ filter (Schleicher and Schull, Dassel, Western Germany). The IgG fraction of the antiserum (MARG) was prepared by ammonium sulfate precipitation and, after dialysis, purified by column chromatography on Sepharose-4B-CL-coupled protein A. The eluates were dialyzed, neutralized, and concentrated to their original cytotoxic titer. Lymphocytotoxicity of the antiserum was assessed in a trypan blue exclusion assay. The cytotoxic titer of the MARG was 1 : 16,000.

Goat anti-mouse lymphocyte serum (GAMLS) was prepared by a subcutaneous injection of 10^8 C57Bl/10 lymphocytes in CFA into a goat, followed by weekly intravenous boosts of the same number of C57Bl/10 lymphoid cells. The serum obtained 1 week after the fifth immunization was heated at 56 C for 45 minutes, and, after precipitation with ammonium sulfate, dialysis, concentration to the original volume, and sterilization, stored at −20 C.

Transplantation Procedure

Squares of 0 8 × 0 8-cm full-thickness skin from male PVG/c tails were grafted onto the flanks of male C57Bl/10 recipients by a modified fitted graft technique [4] The fate of the grafts was followed by daily macroscopic examination Grafts were considered to be rejected when no viable epithelium remained Median survival times (MST) and standard errors were calculated according to the method of Litchfield [18] To postpone cellular rejection of the grafts, we treated all recipients with GAMLS on Days −2, −1, 0, 2, and 4 after grafting in a dose of 0 25 ml intraperitoneally This immunosuppressive regimen prolonged the MST of the PVG/c skin grafts to 27 0 ± 1 1 days

Acute Rejection Studies

On Day 7 after grafting, when all grafts were in excellent condition and showed no signs of rejection, the recipients received MARG via an intravenous injection into the tail vein Macroscopic aspects and histologic features of the resulting graft destruction were then studied AAR was defined as the complete necrosis of the graft within 24–72 hours after the intravenous injection of MARG

Complement Depletion

Complement depletion was induced by COVF, 100 U/ml (Cordis Ltd, Miami, Fla) This preparation was administered from Day 5 until Day 9 after transplantation in daily intraperitoneal injections of 20 U We have previously shown that this dose regimen lowers the serum complement activity to unmeasurable levels from 24 hours after the first injection [7] On Day 7 after transplantation the intraperitoneal injection of COVF was given 2 hours before the intravenous injection of MARG

Complement Suppletion

Fresh frozen serums from rabbits, stored at −70 C, were used as a source of complement (RC) To make certain that the rabbit serum was not a source of antibody activity against the rat xenograft, we absorbed it with PVG/c lymphoid cells One ml of packed cells was incubated with 5 ml RC during 10 minutes at 4 C Both before and after this incubation, no cytotoxic activity of the RC against PVG/c cells could be detected in the trypan blue exclusion assay In the mice that received complement suppletion, the RC was injected intravenously, together with the antibody, in a dose of 0 25 ml In control experiments absorbed RC was used after heat inactivation at 56 C for 45 minutes

Histologic Examination of the Grafts

The skin grafts and surrounding recipient skin were removed at different times after the intravenous injection of MARG and fixed in 4% buffered formalin From paraffin-embedded tissue blocks 4 μ sections were cut and stained with hematoxylin and eosin (H&E), Giemsa, Azan, elastic van Gieson stain, and Mallory's phosphotungstic acid hematoxylin (PTAH) stain for detection of fibrin The presence of fibrin deposits in the Mallory PTAH stain was taken as evidence for the presence of intravascular coagulation Platelet aggregation was assumed to be present when homogeneous concentrations of platelets were seen in a circular distribution along the vessel walls or obliterating the vascular lumen Polymorphonuclear granulocyte (PMN) accumulation was defined as the presence of PMNs in the vessels in numbers exceeding that of erythrocytes and not in evident correlation with platelet aggregation The histologic descriptions were based on findings in at least 3 mice per observation point

Results

Influence of COVF Treatment on the Histologic Features of Acute Antibody Mediated Rejection

Table 1 shows the dose-response relationships of the MARG injections in normal and in complement-depleted recipients In immunosuppressed mice that had a normal complement status, injection of 0 005 ml MARG on Day 7 after transplantation caused a transient edema in the grafts, visible between 2 and 8 hours after injection, but did not lead to acute rejection of any of the grafts (Group 2) The MST of these grafts was 26 9 ± 1 1 days and was not significantly different from the MST of 27 0 days in the control group that received no antibody (Group 1) A dose of 0 01 ml MARG caused total necrosis in about 50% of the grafts within 72 hours after injection (Group 3) The remaining grafts of this group showed, after an initial stage of edema and redness, either a slowly developing deterioration of condition or complete recovery, leading to a MST of 13 5 ± 1 4 days for the group as a whole The histologic sequence of events in the grafts of this group was the same as described in detail in an earlier report [17] Briefly, the acute vasculitis that led to necrosis of the graft after injection

Table 1—Influence of COVF Treatment on the AAR of PVG/c Skin Grafted Onto Immunosuppressed C57Bl/10 Recipients

Group number	Dose of MARG*	COVF† treatment	AAR‡	Histologic features of AAR§
1	–	–	0/12	–‖
2	0 005	–	0/9	–
3	0 01	–	8/19	Arthus-like
4	0 05	–	4/5	Arthus-Shwartzman-like¶
5	0 10	–	9/9	Shwartzman-like
6	0 25	–	10/10	Shwartzman-like
7	–	+	0/8	–
8	0.01	+	0/8	–
9	0 10	+	0/8	–
10	0 25	+	6/11	Arthus-like
11	0 50	+	14/14	Arthus-like
12	1.00	+	8/8	Arthus-like
13	2.00	+	8/8	Arthus-like

* MARG was injected intravenously on Day 7 after grafting.

† Twenty units of COVF was injected intraperitoneally daily, starting on Day 5 after grafting, and was continued for 5 days or until rejection was complete

‡ Number of mice with complete necrosis of the graft within 24–72 hours after MARG injection per number of mice tested

§ Histologic observations made at 30 minutes, 2 hours, 8 hours and 24 hours after MARG injection in groups of 3 mice per point.

‖ Median survival time 27 0 ± 1 1 days

¶ Both types of vasculitis in the same graft

of a dose of 0.01 ml MARG was characterized by early intravascular accumulation of polymorphonuclear granulocytes in the vessels, first seen at 30 minutes after injection and maximal at 2 hours. In later stages, from 8 hours after injection there was increasing vessel wall damage and intravascular coagulation leading to partial necrosis of the graft at 24 hours. This type of vasculitis strongly resembles that of an Arthus reaction. Intravenous injection of doses of 0.10 and 0.25 ml of MARG provoked a more violent reaction in the grafts, with swelling, cyanosis, and bleeding, from 2 hours after injection, and increasing necrosis in all grafts, until no viable epithelium remained after 24–48 hours (Groups 5 and 6).

Histologically, these grafts showed an acute vasculitis that was characterized by early platelet aggregation along the vessel walls, first visible at 30 minutes and extensive at 2 hours. From this time intravascular coagulation was seen, with severe damage of the vessel walls and extensive bleeding. From 8 hours necrosis was prominent, and rejection invariably followed. The sequence of events of in this type of vasculitis closely resembles that of an experimental Shwartzman reaction. As we have mentioned in our previous report,[17] the difference between the Arthus-like and Shwartzman-like types of vasculitis can best be seen in the early stages of the process, since in the later phases intravascular coagulation and necrosis occur in both types.

When we compared the reaction of the grafts to similar doses of MARG in complement-normal recipients with those in the COVF-treated groups, we found that in the complement-depleted animals the sensitivity to MARG was markedly decreased (Groups 8–13). However, all grafts could again be destroyed by the raising of the dose of antibody to 0.50 ml. These results show that acute graft destruction can be induced in recipients that have no measurable amounts of serum complement activity and confirm the findings of our previous study.[7] To study the influences of complement depletion on the histologic sequence of events during the acute graft rejection, we compared the histologic reactions to a dose of 0.25 ml of antibody in complement-normal mice with those in mice treated with COVF. In the complement-normal mice the grafts showed early platelet aggregation along the vessel walls, already visible 30 minutes after injection, accompanied by vasodilatation, hyperemia, incipient endothelial destruction, and extravasation of erythrocytes. Two hours after injection these changes had markedly increased. There was severe endothelial destruction, and intravascular fibrin threads were demonstrable with the Mallory stain (Figure 1A).

At 24 hours the grafts showed total to subtotal necrosis. PMNs did not play an essential role in this process of rejection at any moment. In the first 2 hours after injection of the antiserum no accumulation of PMNs was seen in the graft vessels, except for a slight increase in those areas wherein platelet aggregation was already evident. Also at 8 hours PMNs were only present in small numbers in areas of intravascular coagulation, platelet aggregation, and necrosis.

In the groups that were depleted of complement by administration of COVF, the histologic sequence of events was different. In these mice the first reaction after injection of the same dosè of antiserum (0.25 ml) consisted of a considerable increase of PMNs in the graft vessels, first seen at 30 minutes, and maximal at 2 hours (Figure 1B). At these stages no platelet aggregation or intravascular coagulation could be found, and the grafts showed only minimal edema and congestion. The endothelium of the graft vessels generally showed a moderate swelling of the cells, but no signs of destruction. At 8 hours platelet aggregation and intravascular coagulation were evident, together with vessel wall destruction and marked extravasation of erythrocytes. At this time the grafts showed partial necrosis, varying between 10% to 30%. After 24 hours the histologic changes were not essentially different from those after 8 hours, but the degree of necrosis varied between 10% and 90%. Macroscopic rejection occurred in 6 of 11 animals

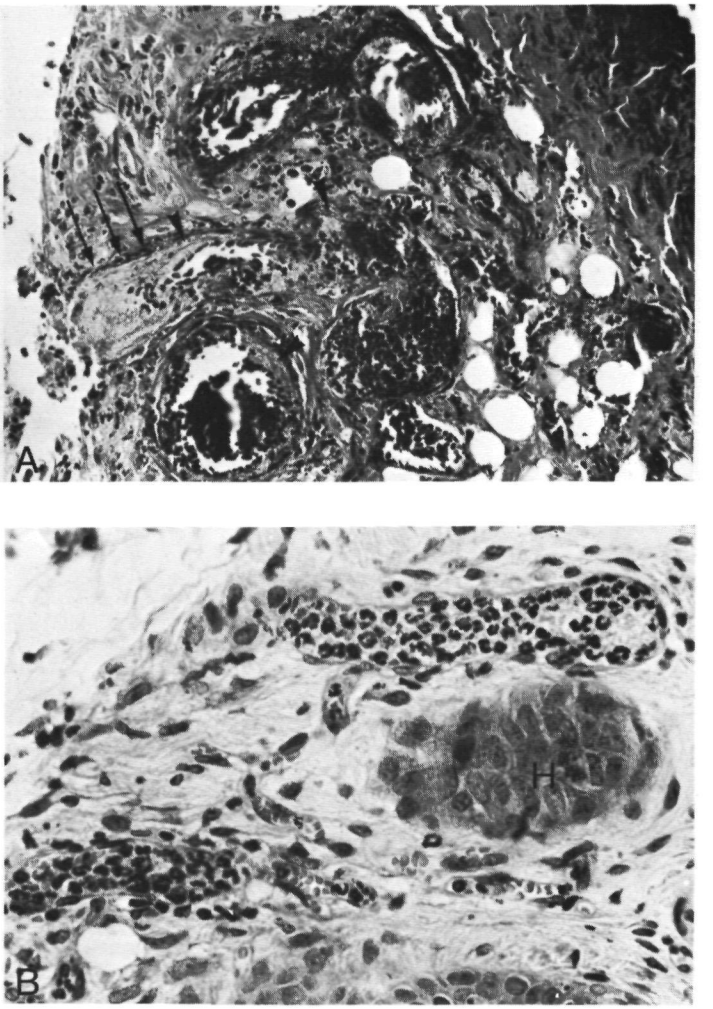

Figure 1—Influence of complement depletion on the histologic pattern of vessel wall damage in AAR. **A**—Pattern in complement-normal mice at 2 hours after injection of 0.25 ml MARG. Extensive platelet aggregation along the vessel walls (*arrows*), in which there are locally visible fibrin threads (*arrowheads*). Destruction of endothelial cells, congestion, and extravasation of erythrocytes (*black*). (Mallory's PTAH, ×78) **B**—Pattern in COVF-treated mice at 2 hours after the same dose of 0.25 ml MARG. Intensive accumulation of PMNs in the vessels. No endothelial cell lysis and no intravascular coagulation at this stage. *H*, hair follicle of the graft. (H&E, ×125)

(Table 1, Group 10) and this rejection developed more slowly than that in the complement-normal group. Partial recoveries were also seen. In the COVF-treated mice the number of PMNs in the vessels at 2 hours after injection of MARG was in general higher than that seen in the Arthus-like reaction of complement-normal mice after low doses of antibody (Figure 1B and Figure 2A). This result is probably related to the higher numbers of circulating PMNs in COVF-treated animals (Table 2). Attempts were made to induce a Shwartzman-like response in COVF-treated mice by a further increase in the amount of injected antibody, but even doses as high as 2.0 ml MARG (given as 0.50 ml of four times concentrated serum) still resulted in an Arthus-like type of reaction. (Table 1, Groups 11–13). Control mice that received COVF alone without MARG (Table 1, Group 7) showed no macroscopic reaction in the grafts and had a MST similar to that of Control Group 1. Microscopically these COVF-treated mice showed on Day 7 after grafting only a minimal increase of PMNs in the vessels, consistent with the general increase in circulating PMNs after COVF treatment.

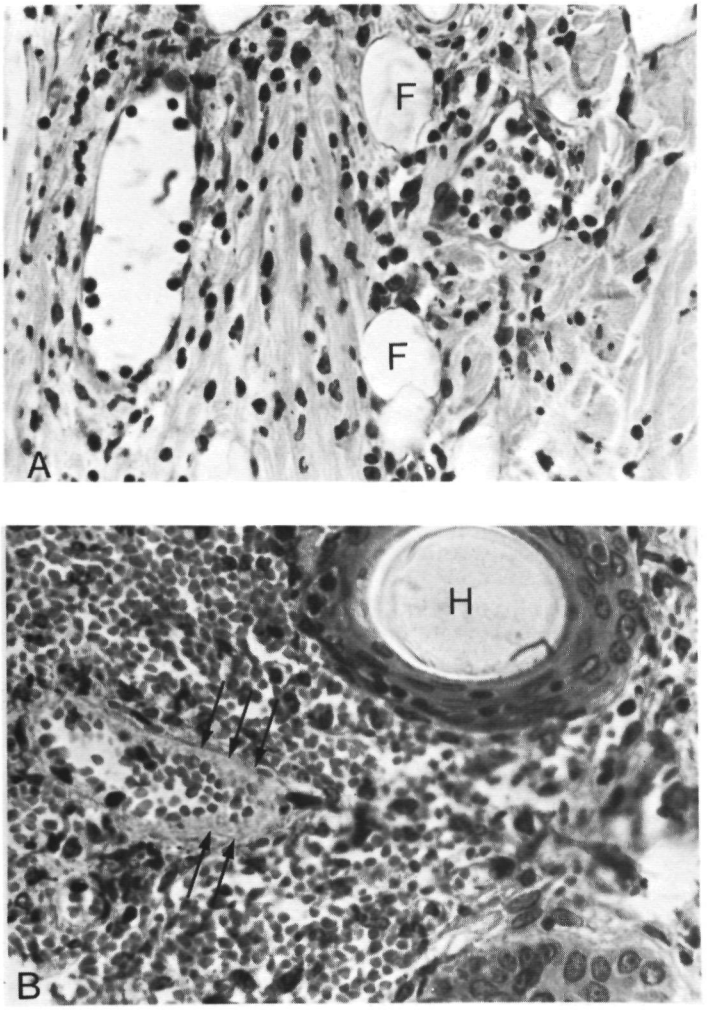

Figure 2—Influence of complement suppletion on the histologic pattern of vessel wall damage in AAR. **A**—Pattern in complement-normal mice at 2 hours after injection of 0.008 ml MARG. Accumulation of PMNs in the vessels. No endothelial cell lysis and no intravascular coagulation at this point. *F*, fat vacuole. (H&E, ×125) **B**—Pattern at 2 hours after injection of 0.25 ml RC together with 0.008 ml MARG. Destruction of endothelial cells, platelet aggregation along the vessel wall (*arrows*), and extravasation of erythrocytes. *H*, hair follicle of the graft (H&E, ×160)

Influence of Complement Suppletion on AAR

The finding that complement depletion diminished the destructive effect of antibody on the grafts suggested the possibility that suppletion of exogenous complement could enhance the destructive effect of subliminal doses of antibody. In our system the limiting dose of MARG that could cause acute antibody-mediated rejection in about 50% of complement-normal mice was 0.01 ml (Table 1, Group 3). We therefore tested the effectiveness of this and two lower doses of MARG, (0.01, 0.008, and 0.005 ml, respectively) when a simultaneous injection of 0.25 ml of RC was given intravenously. Control groups received MARG with heat-inactivated RC or fresh RC alone. The effect on graft survival with and without RC is shown in Table 3. Simultaneous injection of 0.25 ml RC enhanced the effect of 0.01 ml MARG from a rather slowly developing rejection in four of five grafts within 72 hours to a rapid graft destruction in all animals within 48 hours (Group 1). Injection of 0.008 ml MARG with and without RC gave comparable results. A dose of 0.005 ml MARG that normally induced no significant decrease of graft survival (Table 1, Group 2, MST 26.9 ± 1.1 days) also caused after simultaneous injection of 0.25 ml RC, AAR in

Table 2—The Influence of COVF Treatment on the Number of Peripheral Granulocytes in Male C57Bl/10 Mice

	COVF treatment*	PMN/cu mm†
Untreated	–	880 ± 61
	+	3402 ± 432
Immunosuppressed mice carrying rat skin grafts	–	878 ± 222
	+	3667 ± 688

* Daily intraperitoneal injections of 20 U COVF were given during 3 days, and PMNs were counted 2 hours after the third injection. In the transplanted group COVF-treatment was started at Day 5 after grafting.

† Mean values of 4 mice ± SEM.

100% of animals within 72 hours (Table 3, Group 3). The macroscopic experiments were repeated twice, and the results were completely reproducible.

For histologic examination of all three groups and control groups, grafts were removed at 2 hours after the injection of the antiserum. As pointed out before, this is the best moment for determination of the type of vasculitis in progress, especially whether it is Arthus-like or Shwartzman-like. The grafts of mice that received 0.01 ml MARG without RC showed a rejection pattern that at 2 hours was characterized by intravascular accumulation of PMNs with only very sporadic and slight platelet aggregation. Even this minimal platelet aggregation was absent in the group that received 0.008 ml of MARG without RC, and here we saw a pure Arthus-like reaction, with intravascular accumulation of PMNs, but without vessel wall destruction and with only minimal edema (Figure 2A). In the mice that received 0.005 ml MARG without RC the grafts showed some edema and a moderate increase of PMNs in the vessels, but these changes had no substantial effect on the ultimate graft survival. In contrast with these findings, the grafts of the mice that had received RC with 0.01 and 0.008 ml MARG all showed at 2 hours extensive intravascular platelet aggregation, endothelial damage, edema, massive extravasation of erythrocytes, and incipient necrosis, ie, a typical Shwartzman-like reaction (Figure 2B). The reaction in the group that received 0.005 ml MARG with RC was somewhat less severe, macroscopically and histologically, but nevertheless also of the Shwartzman type, with evident platelet aggregation and vessel wall damage at 2 hours. Without the addition of RC, a rapid Schwartzman-like reaction is only seen after intravenous injection of doses of at least 0.10 ml MARG (Table 1, Group 5). Thus, administration of RC together with low and subliminal doses of MARG not only increased the destructive effect of the antiserum with a factor greater than 20, but it also changed the histologic sequence of events from an Arthus-like pattern of rejection, in which mediation of PMNs seems essential, to a Shwartzman-like pattern, caused by immediate endothelial destruction.

Discussion

Clinical and experimental studies have shown that the histologic patterns of the acute antibody-mediated graft rejection closely resemble those seen in other types of immune complex injury[2-7 11-17] and that complement and PMNs can play an important role as secondary mediators. It is therefore not surprising that attempts have been made to prevent destruction by elimination of these mediators. With regard to the effect of complement depletion, these studies have given contradictory results.[6 7] Our study might explain these apparent contradictions because it shows that the effects of complement are only interpretable if there is insight into the amount of antibody available for binding to the corresponding antigen. When the amount of antibody injected is limited, and only sufficient to cause little or moderate damage to the graft, the destructive effect can be greatly enhanced by addition of an efficient complement source to the system (Table 3). However, at high doses of antibody the role of complement becomes minor or even completely superfluous, since AAR can then be induced in COVF-treated recipients that have no detectable complement activity. It is obvious that the concentration of antigen, ie, the degree of antigenic difference

Table 3—Influence of Simultaneous Intravenous Administration of 0.25 ml RC Together with Liminal and Subliminal Doses of MARG on the AAR of PVG/c Skin Grafted Onto Immunosuppressed C57Bl/10 Recipients

Group number	Dose of MARG (ml)*	RC†	AAR‡	Histologic features of AAR§
1	0 01	–	4/5	Arthus like‖
	0 01	+	5/5	Shwartzman like
2	0 008	–	2/4	Arthus-like
	0 008	+	4/4	Shwartzman-like
3	0 005	–	0/5	–¶
	0 005	+	4/4	Shwartzman-like
4	–	+	0/5	–

* MARG was injected on Day 7 after grafting

† The RC was absorbed with PVG/c lymphoid cells (see Methods) The control groups received the same absorbed RC after heat inactivation (–)

‡ Number of mice with complete graft necrosis within 24–72 hours after MARG injection per number of mice tested

§ Histologic features of AAR as seen at 2 hours after injection of MARG in three grafts per group

‖ In two of the grafts there was sporadic platelet aggregation

¶ Transient edema and increase of PMNs in the vessels, not significantly affecting the ultimate graft survival

between graft and host, is important as a third factor that determines the sensitivity of the graft to destruction by antibody This factor is not a limiting one in the model that we have used here, because the xenogeneic difference makes it likely that antigenic targets are available in excess However, in allogeneic models, in which the genetic difference is much smaller, antigen can become the limiting factor This is clearly illustrated by our previous finding that AAR in allografts cannot be induced by antibody alone, even when high doses are injected In these models the addition of an efficient heterologous complement, together with the antibody, is necessary for one to obtain sufficient damage to the vessel walls for AAR to occur [2-5] The fact that in the xenogeneic model AAR can be induced without the addition of heterologous complement has made this model suitable for the study of the role of complement, ie, the study of the effect of complement depletion on the one hand and of complement suppletion on the other

Histologically, AAR, as a type of immunologically mediated vasculitis, can develop either as a Shwartzman-like or as an Arthus-like reaction In a previous study we showed that the histologic pattern can be altered from an Arthus-like into a Shwartzman like reaction by a simple increase in the amount of admin istered antibody [17] The results of the current study demonstrate that addition of an efficient heterologous complement can have a similar effect (Table 3, Groups 1 and 2) It enhances the destructive power of low doses of antibody to such extent that immediate endothelial cell lysis will occur At that moment the exposure of subendothelial tissues to the blood flow will cause early platelet aggregation, without mediation of PMNs, and rapid necrosis of the graft will take place along a Shwartzman-like pattern If no heterologous complement is added to the system the reaction will be dependent on the antibody in cooperation with the much less efficient mouse complement The total destructive power will then be insufficient to cause immediate lysis of the endothelial cells, but chemotactic factors released by the immune reactants and by the moderately damaged cells will attract PMNs, which subsequently, by release of their lysosomal enzymes, act as mediators in the amplification of the destructive process Thus, the intravascular sequence of events will be similar to that of an Arthus reaction These findings bring Arthus-like and Shwartzman-like types of acute rejection together into one spectrum of antibody-mediated vasculitis They suggest that apparent controversies in the literature about the pathogenesis and histologic features of immune vasculitis can be related to differences in concentrations of immune reactants or secondary mediators in the respective experimental systems

A point of interest in our study is that in the Shwartzman-like pattern that follows the injection of high doses of MARG or the injection of low doses of MARG combined with rabbit complement, the mediation of PMNs is apparently not essential for endothelial destruction, because PMNs are not seen in increased numbers in the vessels in the early phases of the process Nevertheless, high amounts of chemotactic factors must be released as soon as the antibodies reach their targets That these chemotactic factors do not cause early accumulation of PMNs in the vessels can be explained as an effect of the early intravascular coagulation that obstructs the capillary lumens, especially at the base of the graft In later stages of the process, when severe extravasation of erythrocytes has taken place, followed by necrosis, PMNs can be seen in increased numbers in the interstitium However, it is obvious in the histologic sections that these PMNs do not reach the graft via the obstructed graft vessels but are conveyed by the host vessels, which they leave at the demarcation line of the graft to move further via the interstitial tissues

A surprising finding was that depletion of complement by COVF treatment caused a histologic type of vasculitis with the characteristics of an Arthus reaction This seems not compatible with the generally held view that fixation of complement is an essential step in the Arthus reaction, because, by its activation, chemotactic substances are generated that attract PMNs It is not clear how in our system of C-depleted recipients the initial leukotaxis is generated The primary attraction of PMNs can be the result of immune adherence of the PMNs to the Fc part of the antibodies, bound to the vascular endothelium It has been shown that such adherence can occur without involvement of complement [19] As soon as the PMNs release lysosomal enzymes, either during phagocytosis[20] or independently from phagocytosis,[20-23] more chemotactic factors will be generated in a positive back loop system in which not only the enzymes themselves but also the damaged cells are participants [24] In our system the effect is probably enhanced by the granulocytosis in the peripheral circulation caused by the COVF treatment (Table 2) This effect of COVF treatment has been reported before,[25] as has been the finding that PMNs can accumulate at cutaneous Arthus sites after COVF treatment [9 25] It is possible that apart from inducing granulocytosis, COVF treatment may change the membrane proper-

ties of the PMNs directly in such a way that adhesion to exposed Fc parts is facilitated. There are, however, no data available to substantiate this hypothesis.

One could argue that the occurrence of AAR in complement-depleted mice was caused by traces of complement activity not measurable in the hemolytic complement assay but still present after treatment with COVF. This residual activity might then have been sufficient to trigger the reaction after injection of a high dose of antibody. However, our previous finding that high doses of non-complement-fixing IgG1 antibodies also caused AAR not only in mice with a normal complement status but also in COVF-treated mice argues strongly for the involvement of a mechanism that is completely independent of complement activation.[7]

The finding that in COVF-treated recipients doses of MARG up to 2.0 ml could only elicit an Arthus-like type of rejection shows that even very high doses of antibody cannot effectuate immediate endothelial cell lysis unless complement is activated. Apparently in the complement-depleted system early intravascular coagulation cannot occur as an initial event in the AAR.

The differences in Arthus-like and Shwarzman-like patterns were most clearly present at the early stages of the process. Once necrosis of the graft had started, intravascular coagulation and PMN accumulation were seen in both types of rejection. Furthermore, even at early stages we sometimes found Arthus-like and Shwartzman-like reactions in the same graft when an intermediate dose of antibody had been given (Table 1, Group 4). Since small differences in concentration or activity of the immune reactants may completely alter the histologic picture, it may be very difficult to assess the actual destructive power of the different factors in clinical studies or in experimental models wherein no inbred animals are used. It is, therefore, not surprising that the findings in these situations are often controversial.

In conclusion, our results show that complement can greatly amplify the immune vasculitis caused by antibody, while modifying its histologic pattern. On the other hand, the presence of complement is not absolutely required for the rejection, since AAR can still be induced in C3-depleted mice that have no measurable serum complement activity. Our finding that Arthus-like and Shwartzman-like types of graft rejection are part of one spectrum of antibody-mediated vessel wall damage, in which the total of the destructive power of the combination of antibodies and complement determines the sequence of events to occur, may lead to a better understanding of the pathogenesis not only of the clinical and experimental graft rejection but also of the related forms of immune complex vasculitis.

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CHAPTER 4

TOTAL BODY IRRADIATION AS A METHOD TO INDUCE GRANULOCYTE DEPLETION IN MICE

M.J.J.T. Bogman, I.M.H.A. Cornelissen, J.H.M. Berden, J. de Jong*, and R.A.P. Koene

*Department of Radiotherapy

ABSTRACT

Since conventional methods to induce depletion of polymorphonuclear granulocytes (PMNs) in mice, such as treatment with cytostatic drugs and anti-PMN sera, proved to be insufficient to induce a stable PMN depletion for several days, and were accompanied by considerable toxic side effects, we induced neutrophil depletion in mice by Total Body Irradiation (TBI) in a single dose of 6.0 Gy (600 rad) at a dose rate of 0.20 Gy min^{-1}. This treatment reduced the number of PMNs in the peripheral circulation to values below 150/µl from day 3 to 10 after irradiation. The number of lymphocytes dropped simultaneously with that of PMNs. Platelet counts remained above 60% of normal values in the first 7 days after irradiation. Complement levels were not significantly affected by TBI. Our results show that a TBI of 6.0 Gy induces a pronounced and stable PMN depletion in mice for at least 7 days. Furthermore, under an aseptic regimen the mice can be kept in good condition and losses are less than 5%.

INTRODUCTION

Studies on the role of polymorphonuclear granulocytes (PMNs) in inflammatory and immunologic reactions in vivo often require the induction of PMN depletion in the animals under study. Conventional methods to reach this goal have consisted of treatment with cytostatic drugs, such as nitrogen mustard (Stetson and Good, 1951; Cochrane et al 1959; Hawkins and Cochrane 1968; Movat et al 1968), or treatment with antigranulocyte serum (Cochrane et al 1965; Hawkins and Cochrane, 1968; Simpson and Ross, 1971; Forbes et al, 1976; Salant et al, 1980). Treatment with nitrogen mustard (HN2) has been reported to be especially effective in rabbits (Stetson and Good, 1951; Cochrane et al, 1965; Movat et al, 1968). In rats PMN depletion is generally induced with anti-PMN serum, but the effect does not seem to be very reproducible (Cochrane et al, 1965; Forbes et al, 1976). We have not found reports of satisfactory results of either treatment with cytostatic drugs or anti-PMN serum in mice, and our own results using these methods in a model, that demanded a PMN depletion in mice to granulocyte counts below 150/µl for a period of at least 5 days, have been unsatisfactory. Therefore, we have used Total Body Irradiation (TBI) as a method to effect neutrophil depletion in mice. The present report describes the effect of TBI on blood cell counts in mice, as compared with that of conventional methods used to induce PMN depletion.

MATERIALS AND METHODS

Animals. Inbred strains of C57Bl/10 mice, originally obtained from the Jackson Laboratory (Bar Harbor, Maine), were kept in our laboratory by continuous brother-sister matings. The mice were used for the experiments at the age of 3-4 months. New Zealand white rabbits or goats were used to prepare anti-granulocyte serum.

Depletion of PMNs. To induce PMN depletion in mice three methods were employed:

A. Treatment with nitrogen mustard (mechlorethamine hydrochloride, HN2). Mice received i.p. injections of nitrogen mustard (Mustini HCl, Brocacef BV, Maarssen, The Netherlands) at a dose of 1.75 mg/kg, on 4 subsequent days. To prevent infection the mice received daily Terramycin (oxytetracycline, Pfizer) orally, in a dose of 7.5 mg (0.15 ml), starting 2 days before treatment, and were kept in a laminar flow unit under aseptic conditions.

B. Treatment with antigranulocyte serum. Anti-mouse granulocyte serum was prepared with a modification of the method described by Cochrane et al (1965). In short: 4ml of a 0.1% w/v solution of glycogen in 0.15M saline was injected i.p. in mice. Four hours later the peritoneal cavity was washed with 7ml heparinized (500 U/ml) saline. Thus obtained peritoneal exudates of 60 mice were pooled and the cells were washed twice with phosphate buffered saline (PBS). Contaminating erythrocytes were lysed with tris-ammoniumchloride 0,83%, and most of the platelets were eliminated by discarding the supernatants after repeated centrifugations at 100g. The number of cells obtained after terminal washings in PBS was $1.5\text{-}2.4 \times 10^8$, and the percentage of granulocytes was 65-70%. Attempts to enrich the suspension of PMNs by means of gradient centrifugation failed because of considerable cell losses in this procedure, due to a large overlap in density between mouse granulocytes and lymphocytes. The cells were resuspended in 5 ml Ringer's solution, and, after mixture with 5 ml complete Freund's adjuvant (CFA), injected s.c. in a rabbit or a goat. When rabbits were used a second immunization followed after four weeks, by i.v. injection of 2×10^8 cells, and 7 days later the animals were bled. In goats the first s.c. immunization was followed by 5 weekly i.v. boosts of the same number of cells, and the animals were bled 7 days after the last immunization. Because in rats the peritoneal exudates, obtained by peritoneal washings after previous i.p. injection of 0.1% glycogen, contain 95% granulocytes, we also prepared anti-rat PMN serum in rabbits and goats, by repeated immunizations with 2×10^8 cells as described above. Part of the anti-PMN sera was absorbed with mouse erythrocytes, thymus and lymph node cells, platelets, and plasma, using 1 vol of packed cells and 0.2 ml of mouse plasma to 10 ml of antiserum. After sterilization by passage through a sterile 20mμ filter (Schleicher and Schüll, Dassel, Germany) the sera were stored at $-20^{\circ}C$. Mice that received anti-PMN serum were kept in a laminar flow unit and received antibiotic treatment as described under A.

C. Total Body Irradiation. To effect PMN depletion the mice received a Total Body Irradiation (TBI) of 6.0 Gy (600rad). The irradiation was performed with an orthovolt X-Ray-unit (Stabilipan, Siemens) at 250 kV, filter Thoraeus II, resulting in a half value layer of 3.1 mm copper. The dose rate was 0.20 Gy min^{-1}, and the field size 15x20 cm^2, at a

focus-skin distance of 76.6 cm. Before undergoing TBI the mice were anaesthetized by an i.p. injection of sodium pentobarbital, 0.03 mg per g body weight (Narcovet, Apharma BV, Arnhem, The Netherlands) and placed between two perspex plates, the upper one being 2 mm thick. To minimize intestinal complications the TBI was preceded by 24 hr of fasting. To prevent infection the mice were kept under aseptic conditions in a laminar flow unit, from 2 days before until 21 days after the irradiation, while they received Polymyxin B (40 mg/l) and Neomycin (100 mg/l) in their drinking water, and heat sterilized food.

Cell counting. Leukocytes in blood drawn from the retrobulbar plexus were either counted by a manual method, using a Pürker Hemocytometer, or in an automatic electronic cell counter (Sysmex Toa). Manual counts were done in fourfold. The differences between the mean values counted by the electronic and the manual method, simultaneously performed in 6 groups of 3-4 mice (at day 4 after TBI of 6.0 Gy) were less than 10% and within the error range of both methods. Differential counts were performed on May Grünwald Giemsa stained air-dried smears. If possible 200 cells were counted but in treated and especially in irradiated mice these numbers were not always present in the 2 or 3 blood smears of one mouse. In the studies on the effect of HN2 and antigranulocyte serum, platelets were counted in a Bürker Hemocytometer using Feissly fluid for dilution. In the mice that received TBI, platelet counts were performed in a Thrombocounter C (Coulter).

Complement assay. Serum complement activity was measured in a sensitive hemolytic assay using the method of Berden et al (1978).

RESULTS

Treatment with Nitrogen Mustard. The effects of treatment with Nitrogen Mustard (HN2) on the blood cell counts of the mice, after i.p. injection of a dose of 1.75 mg/kg on 4 subsequent days, are shown in Table I. At 24 hr after the 3rd and 4th injection the number of PMNs were below 150/µl in only 4 out of 7 mice. At 48 hr after the fourth injection the number of PMNs had already risen to 20-50% of normal values in all mice. Meanwhile, repeated injections caused increasing toxic side effects and death losses were more than 20%. When the dose of HN2 was raised to 3.5 mg/kg PMN counts lower than 100/ l could be obtained for a period of 24 hr after the fourth injection, but the surviving mice were too ill to be

Table I: Effect of treatment with Nitrogen Mustard, at a dose of 1.75 mg/kg i.p. given on subsequent days, on the blood cell counts of adult male C57Bl/10 mice

No of i.p. Injections	Time between Last Injection and Cell Counting (hr)	Leukocytes /µl	PMNs /µl	Platelets x 10^{-3}/µl
1	24	5200, 3000	312, 210	235, 240
3	24	3800, 3600 2600, 1400	152, 72 78, 42	705, 640 630, 725
4	24	3600, 1500 2000	72, 180 240	580, 590 790
4	48	7700, 2600 5100, 3900	580, 260 204, 350	1060, 985 990,1070
Controls (n=6)		10,200±1600	1255±365	1120±250

used for further experiments. As our experimental model required a stable PMN depletion to levels below 150/µl for at least 5 days, during which the mice had to remain in good condition, we considered treatment with HN2 unfit to reach this goal. As shown in Table I platelet counts were moderately affected by treatment with HN2. Following an initial dip to 20% their numbers remained at 50-70% after repeated injections, with a quick recovery at 48 hr after the fourth treatment. Mononuclear cell numbers were reduced approximately parallel to those of granulocytes.

Treatment with anti-granulocyte sera. Several pools of anti-PMN serum were used, and we varied the treatment from doses of 0.5 ml i.p., given at 16 to 18 hr and 2 to 4 hr respectively before cell counting, to doses of 1.0 ml twice a day on 3 subsequent days. However, even the high doses of the most efficient antisera did not induce a PMN depletion in which granulocyte levels were constantly below 150/µl in all animals. An illustration of representative effects of two doses of several pools of antiserum is given in Table II. The most effective anti-mouse-granulocyte serum (RAMGS-I) caused considerable toxic side effects, that were even more severe

Table II. Effect of treatment with different pools of anti-granulocyte serum on blood cell counts of C57Bl/10 mice

Serum	Dose[1] (ml)	Time between Last Injection and Cell Counting (hr)	Leukocytes /µl	PMNs /µl	Platelets x 10^{-3}/µl
RAMGS-I[2]	0.5	4	325	130	520
unabsorbed		24	2600	260	420
RAMGS-I	0.5	4	1200	120	350
absorbed	1.0	4	1200	168	-
RAMGS-II[2]	0.5	4	2900, 2500	780, 750	379, 292
unabsorbed	0.8	4	6100, 2800	1342, 700	248, 370
GAMGS[3]	1.0	2	4800, 7000	1440,1050	198, 486
unabsorbed		4	2700, 5900	890, 760	692, 110
RARGS[4]	1.0	2	2600, 3100	470, 590	498, 598
unabsorbed		4	2000, 1700	200, 340	674, 800
Controls (n=6)			10200±1600	1255±365	1120±250

1) Two injections were given, the first 16-18 hr before the second.
2) Rabbit-anti-mouse-granulocyte serum, pool I and II.
3) Goat-anti-mouse-granulocyte serum.
4) Rabbit-anti-rat-granulocyte serum.

after absorption, leaving only one of four mice alive at 6 hr after the second injection. Since a similar effect was seen after absorption of another pool, and since absorption did not significantly alter the effect on lymphocyte and platelet counts, we used unabsorbed sera for further tests. Although the sera of the rabbits, used for immunization, were previously screened for the absence of preexistent toxicity by injection into mice (0.25 ml i.p. in 3 mice per rabbit), all rabbit antisera proved to be toxic. After two days of treatment the mice had cold fluffy skins with signs of dehydration, and losses were more than 60%. The antisera prepared in goats were in general less toxic, but also less efficient. The unabsorbed as well as the absorbed antisera caused variable degrees of lymphopenia and reduction in platelet numbers (Table II). In 4 mice, treated with rabbit anti-rat-PMN serum, we measured the effect on complement activity in the serum. The CH50 titers in these mice had decreased to values lower than 10% of those of age and sex matched controls (data not shown).

Table III. Effect of TBI of 6.0 Gy on differential blood cell counts and platelets in adult male C57Bl/10 mice

Days after TBI	No. of mice	PMNs/µl ± SD	Mononuclear cells/µl ± SD	Platelets $x10^{-3}$/µl ± SD
3	5	44 ± 16	143 ± 41	1290 ± 207
4	5	18 ± 5	82 ± 5	1190 ± 246
5	5	37 ± 17	146 ± 33	1005 ± 214
6	4	66 ± 17	134 ± 17	781 ± 47
7	3	48 ± 24	152 ± 24	736 ± 96
10	6	25 ± 16	161 ± 46	44 ± 19
Controls	6	1255 ± 365	8865 ± 1487	1169 ± 176

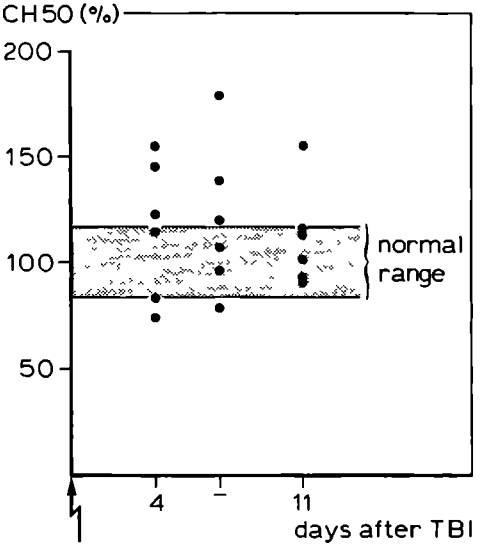

Fig. 1: The effect of TBI of 6.0 Gy on serum complement levels of adult C57Bl/10 mice, calculated as the percentages of the CH50 values of age and sex matched controls.

The effect of Total Body Irradiation: As shown in Table III the effect of TBI of 6.0 Gy caused a stable depletion of PMNs to levels below 100/µl for a period of 3 to 10 days after the irradiation. Furthermore, under the described aseptic regimen and antibiotic treatment the mice could be kept in a healthy condition, with a mortality of less than 5%. TBI in lower doses of 5.0 or 4.0 Gy caused a lesser degree of PMN depletion to values of 177 ± 31 (n=3) and 170 ± 46 (n=4) per µl respectively on day 4 after the irradiation. Parallel to the depletion of PMNs, after TBI of 6.0 Gy a similar depletion of mononuclear cells was seen (Table III). The separate effects on lymphocytes and monocytes were not tested, mainly because the differentiation between these cells in the smears, which is normally already rather difficult, was further impaired by moderate degrees of nuclear pycnosis, due to the irradiation. The overall impression was that the percentage of monocytes among the mononuclear cells, normally amounting to 5-10%, was unaltered by the TBI. Although platelets were reduced in number from day 6 after TBI, their counts remained above 60% of normal values in the first 7 days. A more substantial depletion was reached in the following days, proceeding to a level of about 5% of normal values on day 10 (Table III). The effect of TBI on serum complement levels was tested in 3 groups of 6 otherwise untreated mice on days 4,7, and 11 after irradiation. The levels were calculated as percentages of the CH50 values of age and sex matched controls (fig. 1). The results demonstrate that in contrast to treatment with anti-PMN serum, TBI did not reduce the complement levels.

DISCUSSION

Our results show that for the induction of PMN depletion in mice TBI of 6.0 Gy is the method of choice. Major advantages of this approach as compared to treatment with cytostatic drugs or antigranulocyte serum are: 1) The effected PMN depletion is severe, consistent and stable, and lasts for a period of at least 7 days. 2) By keeping the mice under aseptic conditions and by protective treatment with antibiotics the animals can be kept healthy and active, with few losses, and subsequent procedures, such as intravenous injections for experimental purposes, can be easily performed. 3) During the critical period of PMN depletion no injections are required to keep the PMNs at low levels, which diminishes the risk of infection. 4) TBI does not significantly affect serum complement levels. 5) TBI is relatively easy to administer and time consuming immunizations and absorptions of antisera are not required.

Side effects of TBI are the concomitant depletion of

lymphocytes and platelets. The effect on platelet counts can be largely circumvented by choosing day 4-7 after TBI as the time for experimental procedures, since in this period platelet counts remain above 60% of normal values. The number of lymphocytes, however, drops simultaneously with that of PMNs. In experimental situations in which lymphocyte depletion may be critical, reconstitution with isogeneic lymphoid cells immediately after TBI could, at least in part, compensate for the loss of lymphocytes. An undesirable lowering of lymphocyte and platelet counts was also a side effect of the treatment with HN2 and anti-PMN sera. Major disadvantages of these conventional methods, however, are the inconsistent effect on the PMN levels and the severe toxicity. In all treatments that cause illness of the mice, it has to be kept in mind that dehydration and shock of the animals substantially impair the peripheral circulation. This effect can be critical when the experimental challenge concentrates on organs depending on the peripheral circulation such as the skin. Illustrative for this effect are our findings in an experimental model in which we tested the role of PMNs in the acute antibody mediated rejection of skin xenografts in mice (in preparation). We found that treatment with rabbit anti-rat-PMN serum prevented the rejection, although the PMN depletion was insufficient (200-400 PMNs/µl). To exclude a non specific reaction we repeated the experiments with mice that received anti-lymphocyte serum instead of anti-PMN serum, in similar high doses, that also caused moderate illness in the mice. We found the same preventing effect on the rejection, although the PMN counts remained normal. We concluded that the results of the treatment with anti-PMN serum, that falsely suggested a PMN dependency of the antibody-mediated rejection, were either due to an impaired peripheral circulation, thus preventing the antibody to reach the graft in sufficient amounts, or to a decrease in complement levels, caused by the anti-PMN serum. This assumption was supported by the finding that in mice that received TBI the rejection was not prevented, despite severe PMN depletion.

Theoretically, the most attractive aspect of treatment with anti-granulocyte serum is its specificity. However, the preparation of effective, specific, and non-toxic anti-mouse-PMN serum has proved to be very difficult. The procedure is impaired by the fact that mice in general have very low PMN counts as compared to other animals such as rats or rabbits, and that it is not possible to induce peritoneal exudates that contain 90% or more PMNs like in rats. The overlap in density between mouse lymphocytes and granulocytes further impedes purification of the suspension to be used for immunization. Moreover, absorption of the anti-PMN sera with mouse lymphocytes and platelets did not prevent the depressive effect on lymphocyte and platelet levels. A similar finding has been reported by Simpson et al (1971), about the effect of

absorbed anti-guinea-pig-PMN serum on the lymphocyte levels of the guinea-pigs. In this study the effect on platelet counts was not measured. The effect of anti-PMN serum on the serum complement levels seems to be variable. In our experiments we found a decrease to values below 10% in 4 mice tested. Others reported normal complement values in a similar experimental protocol (Winn et al, 1973). However, it is to be expected that repeated injections with high doses of any effective conventional antiserum will cause concomitant fluctuations in the complement levels of the animals. This effect can become critical if the model is used for the study of immunologically induced inflammatory processes.

We conclude, that for PMN depletion in mice TBI is a relatively easy and very reliable method, that should be considered the method of choice especially in those experiments in which a concomitant depletion of lymphocytes is acceptable.

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CHAPTER 5

ACUTE ANTIBODY-MEDIATED REJECTION OF SKIN GRAFTS WITHOUT INVOLVEMENT OF GRANULOCYTES OR COMPLEMENT

M. José J.T. Bogman, Ine M.H.A. Cornelissen
and Robert A.P. Koene

ABSTRACT

In immunosuppressed mice that carry rat skin xenografts acute antibody-mediated graft rejection (AAR) can be induced by intravenous administration of mouse anti-rat globulin. Dependent on the amount of antibody injected and on the complement status of the recipient an Arthus-like or a Shwartzman-like pattern of vasculitis occurs. The role of polymorphonuclear granulocytes (PMNs) in either type of vasculitis was tested by inducing AAR in recipients depleted of PMNs by Total Body Irradiation. Despite the absence of PMNs in the graft vessels, AAR occurred both in the Arthus-like and in the Shwartzman-like type. Moreover AAR could be elicited in PMN depleted recipients that were complement depleted by Cobra Venom Factor treatment or were congenitally C5 deficient. We conclude that neither PMNs nor complement are essential mediators in this form of antibody-mediated vasculitis.

The characteristic presence of large numbers of polymorphonuclear granulocytes (PMNs) in various types of immune vasculitis has suggested an important role for these cells as mediators in immunologically induced vessel wall damage. The first experimental model in which this role was extensively studied was the Arthus reaction and in this model PMNs proved to be essential mediators in the induction of endothelial damage (1-3). Acute vasculitis with intravascular accumulation of PMNs is also present in grafts that are acutely rejected by circulating antibodies directed against the graft antigens. This similarity to the Arthus reaction (4-7) led to the hypothesis that PMNs were also essential in the acute antibody-mediated graft rejection (AAR). However, studies that tried to prove this have given contradictory results (8-10).

Since in the acute antibody-mediated graft rejection also patterns of vasculitis have been described that resemble the experimental generalized Shwartzman reaction (4, 6, 7, 11, 12) we have thought that some of the controversies could possibly be explained by the fact that the morphological sequence of events in the AAR can vary, and that the role of PMNs may be dependent on the way wherein the initial damage to the endothelial cells takes place. In previous studies on skin allografts and xenografts in mice we have shown that the pattern of acute antibody-mediated rejection is indeed variable and can either resemble an Arthus reaction, in which PMNs seem to play an important role in the first phases of the antibody-induced vasculitis, or can be consistent with a Shwartzman-type of vasculitis, that is characterized by early intravascular coagulation, and in which PMNs morphologically seem to play only a secondary role. Which histological type of rejection will occur after injection of specific antibodies against the donor tissue depends on several factors, such as the immunologic difference between host and graft, the amount of antibodies in the circulation of the recipient, and the availability of complement. When we used an allograft model, in which immunosuppressed mice carrying skin allografts were intravenously injected with specific antibody, acute rejection of the graft could only be elicited when together with the

antibody also exogenous complement was injected. This rejection of allografts always developed along an Arthus-like pattern (13). In a xenograft model, in which immunosuppressed mice carried rat skin grafts, the intravenous injection of anti-rat antibody could readily provoke acute rejection of the grafts without addition of exogenous complement. This rejection of xenografts showed a Shwartzman-like pattern when high doses of antibody were administered, whereas low doses caused a more gradually evolving vessel wall damage, histologically consistent with an Arthus-type of vasculitis (6). Similarly, manipulations in the amount of available complement in this xenogeneic model caused radical changes in the reaction pattern of the grafts. Complement depletion of the recipients changed the reaction in the graft vessels after injection of a high dose of antibody from a Shwartzman-like into an Arthus-like one. Conversely, an Arthus-like reaction could be changed into a more violent Shwartzman-like one when together with a low dose of antibody highly effective rabbit complement was administered (7).

Considering the morphologic differences between the Arthus-like and the Shwartzman-like responses we felt that the role of PMNs should be separately studied for either type of vasculitis. Therefore we chose for our present study the xenogeneic model of rat skin transplanted onto immunosuppressed mice, in which system we can consistently induce either type of rejection by merely varying the amount of injected antibody (6). As in this system AAR can also be elicited in complement depleted or complement deficient mice, provided that the dose of antibody is sufficiently raised (7, 14, 15), we were also able to study the role of PMNs in the absence of complement.

Our results show that PMNs are not essential mediators in the initial damage to the endothelial cells of the graft vessels, neither in the Shwartzman-like nor in the Arthus-like type of AAR. Moreover, we found that even in the absence of complement activity AAR can be elicited in PMN-depleted recipients, although PMNs proved to be important accelerators and amplifiers of the ultimate necrosis of the graft.

MATERIALS AND METHODS

Animals. Inbred strains of C57Bl/10 and B10.D2 old (congenitally C5 deficient) mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine) and inbred PVG/c rats from the Institute of Psychiatry, Bethlem Royal Hospital,

Beckinham (Kent, U.K.). In our laboratory these strains were kept by continuous brother-sister matings. Mice were used for the experiments at ages of 3 to 4 months. Random bred goats were used to prepare anti-mouse lymphocyte serum.

Antisera. Mouse anti-rat lymphocyte serum was prepared in C57Bl/10 mice as described before (6). The IgG fraction of the antiserum (MARG) was prepared by ammonium sulfate precipitation. The lymphocytotoxic titer of the MARG, as assessed in a trypan blue exclusion assay, was 1:26,000. In cases in which more than 0.50 ml of MARG had to be injected an equivalent dose of four times concentrated antiserum was given. The cytotoxic titer of this concentrated pool was 1:120,000; the dose-response relationship in the AAR of rat skin xenografts after four fold dilution of this serum was similar to that of the original pool of MARG.
Goat anti-mouse lymphocyte serum (GAMLS) was prepared as described earlier (7). MARG and GAMLS were heated at 56°C for 45 min to destroy complement activity, sterilized by passage through a sterile 20-mμ filter (Schleicher and Schüll, Dassel, Western Germany), and stored at -20°C.

Transplantation procedure. Squares of 0.8 x 0.8 cm full-thickness tail skin from male PVG/c rats were grafted onto the flanks of male C57Bl/10 or B10.D2 old mice by a modified fitted graft technique (14). The fate of the grafts was followed by daily macroscopic inspection. Grafts were considered to be rejected when no viable epithelium remained. Median survival times (MST) and standard errors were calculated according to the method of Litchfield (16). To postpone cellular rejection of the grafts we treated the recipients with GAMLS on days -2, -1, 0, 2, and 4 after grafting in a dose of 0.25 ml i.p. Two pools of GAMLS were used, that prolonged the MST of the PVG/c skin grafts in C57Bl/10 mice under the above mentioned immunosuppressive regimen to 23.8 ± 1.0 and 24.1 ± 1.1 days respectively, and therefore were considered equivalent. In B10.D2 old recipients the GAMLS treatment as described above prolonged the MST of PVG/c skin grafts to 25.3 ± 1.1 days. In mice that received Total Body Irradiation on day 4 after transplantation the injection of GAMLS on day 4 was omitted. Under these conditions the MST of the rat skin grafts was 24.1 ± 1.0 days in C57Bl/10 recipients and 27.0 ± 1.1 days in B10.D2 old recipients.

Acute rejection studies. On day 8 after grafting, when all grafts were in excellent condition and showed no signs of rejection, the mice received MARG via an i.v. injection into the tail vein. Macroscopic aspects and histologic features of the resulting graft destruction were studied. AAR was defined

as complete macroscopic necrosis of the graft within the next few days. In general this complete necrosis took place within 72 hr, but in the irradiated and complement-depleted mice the progression of graft destruction was slower. This slower type of rejection was only considered to be mediated by the injected antibody if reaction was seen within 24 hr after injection and necrosis was macroscopically complete at least 7 days before the first signs of rejection were visible in grafts of control mice that had not received MARG.

Complement depletion. Serum complement activity was measured in a sensitive hemolytic assay (17). Complement depletion was induced by Cobra Venom Factor (COVF), 100 U/ml, (Cordis Ltd., Miami, Fla), administered from day 6 after transplantation in daily i.p. injections of 20 U. This dose regimen lowers the serum complement activity to unmeasurable levels from 24 hr after the first injection (15). On day 8 after transplantation the i.p. injection of COVF was given 2 hr before i.v. injection of MARG. Treatment with COVF was continued until rejection was complete, or, if no rejection occurred, for maximally 10 days.

PMN depletion by Total Body Irradiation: Mice were depleted of PMNs by Total Body Irradiation (TBI) consisting of a single dose of 6.0 Gy (600 rad) on day 4 after transplantation, at a dose rate of 0.20 Gy min^{-1}, from a 250 kV X Ray source (Stabilipan, Siemens). After 24 hr of fasting the mice were anesthetized by an i.p. injection of sodium pentobarbital, 0.03 mg per g body weight (Narcovet, Apharma BV, Arnhem, The Netherlands) and placed between two perspex plates, the upper plate being 2 mm thick. To prevent a potentially damaging effect of the irradiation on the endothelium of the graft a small piece of lead (12 x 12 x 3 mm), was placed on the upper perspex plate, just above the graft. The side scatter dose on the graft tissue, as measured in a phantom set-up (18) was 20-25% of the total dose of 6.0 Gy. To prevent infection the irradiated mice were kept under aseptic conditions in a laminar flow unit from 2 days before until 3 weeks after TBI, while they received Polymyxin B (40 mg/l) and Neomycin (100 mg/l) in their drinking water, as well as heat sterilized food. This regimen kept the mice in excellent condition throughout the experiment and mortality was less than 10%. Only irradiated mice that also received COVF for several days showed a deterioration of condition which was reversible after withdrawal of the COVF treatment.

Counting of PMNs and Platelets. Leukocytes in blood, drawn from the retrobulbar plexus, were counted in a Bürker Hemocytometer. We compared the manual counts, that were done in fourfold, with simultaneous counts performed on an automatic electronic cell counter (Sysmex ToA).

The differences between the mean values in 6 groups of 3-4 mice were less than 10%, and within the error range of both methods. Differential counts were performed on May Grünwald Giemsa stained air dried smears. Platelets were counted in a Thrombocounter C (Coulter).

Histologic examination of the grafts. Skin grafts and surrounding tissue were removed at different times after the intravenous injection of MARG and fixed in 4% buffered formalin. From parrafin-embedded tissue blocks 4μ sections were cut and stained with hematoxylin and eosin (H&E), Giemsa, Goldner trichrome stain, elastic van Gieson stain, and Mallory's Phosphotungstic acid hematoxylin (PTAH) stain. The histologic descriptions are based on findings in at least 2-3 mice per observation point.

Table I. Effect of TBI (6.0 Gy) on PMN and platelet counts in the peripheral blood of male C57Bl/10 mice

Days after TBI	PMN/μl±S.D.	Platelets x 10^{-9}/l±S.D.	No. of mice
3	44 ± 16	1290 ± 207	5
4	18 ± 5	1190 ± 246	5
5	37 ± 17	1005 ± 214	5
6	66 ± 17	781 ± 47	4
7	48 ± 24	736 ± 96	3
10	25 ± 16	44 ± 19	6
Controls	1255 ± 365	1169 ± 176	6

RESULTS

Depletion of PMNs. Before turning to TBI for PMN depletion we tried to reach this goal by using the earlier published approaches of treatment with anti-PMN sera and cytostatic drugs. Anti-PMN sera were raised in rabbits or goats, according to the method of Cochrane (19) and administered in multiple i.p. injections on the days of and immediately before and after the injection of MARG. The resulting PMN depletion varied with this treatment and could not be

Table II. PMN counts in immunosuppressed[1] and irradiated[2] recipients of PVG/c skin grafts after administration of MARG[3]

COVF[4] treatment	Days after transplantation	Circulating PMN/µl ± S.D. C57Bl/10 PMN depleted	C57Bl/10 Control	B10.D2 old PMN depleted	B10.D2 old Control
-	8	32±24(10)[5]	1474±607(12)	16±16(4)	1500±307(3)
-	9	31±41 (4)	889±318 (4)	42±22(3)	862±382(3)
-	10	46±35 (3)	n.t.[6]	42±12(2)	683±273(2)
-	11	55±21 (3)	n.t.	4± 1(5)	421±382(5)
-	14	30±16 (3)	n.t.	89±22(4)	n.t.
+	8	38±22 (8)	2998±1005(12)	13± 6(5)	1782±465(8)
+	9	48±30 (5)	2007± 244 (3)	25± 9(5)	189± 66(2)
+	10	86±43 (4)	1558 (1)	36±18(5)	537± 86(2)
+	11	24±10 (2)	1748 (1)	20±12(2)	1024±264(2)
+	14	n.t.	n.t.	40±24(2)	554 (1)

1) To postpone cellular rejection the mice received ALS i.p. on days -2, -1, 0, 2, and 4 after transplantation. In mice that were irradiated on day 4 after transplantation the injection of ALS on day 4 was omitted.
2) TBI (6.0 Gy) to induce PMN depletion was given on day 4 after transplantation.
3) MARG (0.01ml in complement normal and 2.0ml in C5 deficient and COVF treated mice) was given i.v. at day 8 after transplantation.
4) 20 U COVF was given i.p. from day 6 -14 after transplantation or until rejection was complete.
5) No. of animals tested given in parentheses.
6) n.t.: not tested.

sustained for the duration of the experiment. This also held for treatment with nitrogen mustard. Furthermore, the mice became increasingly ill after repeated injections of both nitrogen mustard or anti-PMN serum. Extensive absorptions of the anti-PMN serum with mouse lymphocytes, erythrocytes, platelets, and plasma could not prevent this. Lastly, the anti-PMN serum caused considerable decreases in complement levels of the recipients (less than 10% of control mice). We have previously shown that this greatly impairs the sensitivity to AAR (7).

The influence of TBI on the numbers of PMNs in the circulation of otherwise untreated C57Bl/10 mice is shown in Table I. Substantial depletion of platelets did not occur in the first 7 days after TBI (Table I). PMN counts that were performed in mice that were sacrificed for histologic examination of the grafts at different times after the injection of MARG, are shown in Table II. Mice were considered PMN depleted when the number of PMNs in the circulation was less than 150/µl. This condition was reached on day 3 after TBI and remained stable for at least 10 subsequent days in all experiments. The influence of TBI on the complement levels of immunosuppressed mice that carried rat skin grafts was measured in two groups of 6 mice on day 8 after transplantation. The mean values ± SEM of the CH50 titers were 25 ± 5 U/ml and 43 ± 30 U/ml for the irradiated groups versus 38 ± 10 U/ml and 73 ± 27 U/ml respectively for non-irradiated but otherwise similarly treated control groups, corresponding to decreases in complement levels in the irradiated groups to 66% and 59% of control values respectively.

Influence of PMN depletion on AAR in graft recipients with a normal complement status. The effects of i.v. injection of different doses of mouse anti-rat globulin (MARG) in mice carrying rat skin grafts are shown in Table III. Injection of high doses of antibody on day 8 after transplantation

Table III. Influence of PMN depletion on AAR of PVG/c skin grafted onto immunosuppressed C57Bl/10 recipients

Group No.[1]	Dose of MARG (ml)	Occurrence of AAR[2]	
		Control mice	PMN-depleted mice
1	0.25	8/ 8	8/ 8
2	0.10	8/ 8	7/ 7
3	0.01	7/12	9/13
4	0.0075	0/12	1/10
5	0.005	0/ 6	0/ 6
6	-	0/ 8	0/ 9

1) In each dose group age and sex matched mice were treated with the same pool of GAMLS and injected with similar aliquots of diluted MARG in simultaneous experiments.
2) Number of mice with complete macroscopic necrosis of the grafts within 72 hr/number of mice tested.

invariably caused rejection of the grafts within 72 hr, both in PMN-depleted recipients and in control mice (group 1 and 2). At 2 hr after the injection of MARG both groups showed cyanosis of the grafts. In the PMN-depleted mice this reaction was somewhat less intense. Also when low doses of antibody were administered (group 3-5) there were no essential differences in the fate of the grafts between PMN-depleted and control groups. Injection of 0.01 ml of MARG caused a red-blue discoloration of the grafts within 24 hr in both PMN-depleted and PMN-normal mice although the reaction in the PMN-depleted animals was again somewhat less severe. Similarly in the groups that received 0.0075 ml of MARG the control mice showed only slightly more hyperemia of the grafts in the first 48 hr after injection than the irradiated mice. Both groups that received 0.005 ml of MARG showed transient edema and in some cases slight hyperemia of the grafts with no evident difference between PMN-depleted and control mice. In these groups as well as in most of the grafts in the groups that received 0.0075 ml of MARG the reaction was reversible. The MST of these grafts was not significantly different from that in control animals that had not received MARG.

On histologic examination the grafts in the control groups that received high doses of MARG, i.e. 0.25 and 0.10 ml (groups 1 and 2), showed, at 2 hr after injection, vessel wall destruction, intravascular platelet aggregation, and bleeding, consistent with our earlier results (6) (Fig 1 A). At 24 hr there was progression to subtotal or total necrosis of the graft tissue. In the PMN-depleted groups that received 0.25 and 0.10 ml of MARG the grafts showed, at 2 hr after injection of the antibody, also evident vascular damage, platelet aggregation, and extravasation of erythrocytes, although the reaction was somewhat less severe than that in the control groups (Fig. 1 B). Total to subtotal necrosis, with obliteration of the vessels by intravascular coagulation was present at 48 hr. Of the groups that received the low dose of 0.01 ml of MARG the control grafts showed, at 2 hr after injection, vasodilation and hyperemia, accompanied by accumulation of PMNs in the vessels. At 8 hr these changes had increased and platelet aggregation, vessel wall damage, and extravasation of erythrocytes were prominent features. In the PMN-depleted mice that received 0.01 ml of MARG the damage to the vessel walls was strikingly the same, apart from the conspicuous absence of PMNs. Endothelial swelling and vasodilation were seen at 2 hr and vessel wall destruction with local platelet aggregation and bleeding were obvious at 8 hr. As could be expected from the macroscopic observations the vasodilatation and hyperemia were somewhat less in the PMN-depleted group when compared with the control group. The eventual fate of the grafts however, was the same (Table III, group 3).

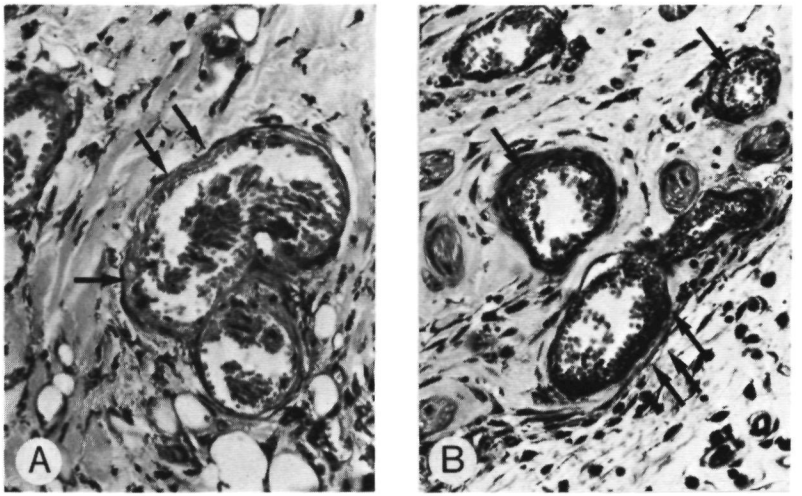

Figure 1. Reaction in the vessels of PVG/c skin grafted onto C57Bl/10 recipients at 2 hr after injection of 0.25ml MARG. Both in control mice (A) and in PMN-depleted mice (B) dilation of the vessels is seen, with platelet aggregation along the vessel walls (arrows), and partial destruction of the endothelial cells. (A and B: Giemsa, 80x).

Influence of PMN depletion on AAR in COVF treated and C5 deficient mice. In an earlier study we have shown that AAR can be provoked in mice that carry rat skin grafts, after C3 depletion with COVF, provided that the dose of antibody is substantially raised (7). We also found that in this complement independent graft rejection the vasculitis that forms the basis of the process, is always of the Arthus type (7). Since we found no significant differences in the occurrence of AAR in PMN-depleted mice as compared to the control groups, even after injection of low doses of antibody that were known to evoke an Arthus-like response in normal recipients, it became important to test whether AAR could be elicited in PMN-depleted mice that were also complement depleted or complement deficient. The results of these experiments are shown in Table IV. In accordance with our previous reports (7, 15) we found that COVF treatment did not prevent AAR after high doses of antibody. In COVF treated mice that were also PMN-depleted by TBI, a dose of 1.0 ml MARG caused a slow and moderate reaction in the first days, that only between days 15 to 20 after transplantation led to total necrosis of the grafts (Group 1). However, if the dose was raised to 2.0 ml total necrosis of 75% of the grafts occurred within 6 days after injection of MARG (Group 2). The reaction was clearly less violent in the PMN-depleted animals than in the control mice. While the control group showed a red-blue discoloration of the grafts within 8-24 hr after injection of

Table IV. Influence of PMN depletion on AAR of PVG/c skin grafted onto immunosuppressed COVF-treated and/or C5 deficient mice

Recipient strain	Group No.	Dose of MARG (ml)	COVF treatment	PMN depletion	AAR[1)]	Days after grafting on which macroscopic necrosis was complete
C57Bl/10	1	1.0	+	-	11/11	9-14
		1.0	+	+	0/9	(15-20)
	2	2.0	+	-	8/8	9-12
		2.0	+	+	11/14	11-14 (19,22,24)[2)]
B10.D2 old	3	1.0	-	-	9/9	9-13
		1.0	-	+	8/8	10-13
	4	2.0	-	-	11/11	9-11
		2.0	-	+	10/10	10-13
	5	2.0	+	-	11/11	9-11
		2.0	+	+	8/10	12-14 (22,26)

1) Number of mice with complete macroscopic necrosis of the graft/number of mice tested.
2) Survival times of grafts that were rejected later than day 14 or that were unaffected by the injected MARG are given in parentheses.

the antiserum, the PMN-depleted group only showed slight hyperemia and edema at 24 hr, with progression to cyanosis after 96 hr. In most of the PMN-depleted mice this cyanosis slowly progressed to total necrosis but some of the grafts showed partial recovery, after which definitive rejection occurred between 19 and 24 days after transplantation. The microscopic events in the grafts of the PMN-depleted, COVF treated C57Bl/10 mice consisted of a slight swelling of the endothelium in the graft vessels at 2 hr after injection of 2.0 ml of MARG, followed by some dilation and hyperemia of the vessels at 8 hr. At 8 hr we also found local platelet aggregation. From this time there was a slow progression of the vasculitis, with local vessel wall destruction and bleeding at 24 hr. The first signs of necrosis were seen at 48 hr. PMNs were absent in the graft vessels at any stage. The COVF treated but non-irradiated control group showed a considerable granulocytosis in the graft vessels during the first hours, followed by platelet aggregation, vessel wall destruction, extensive bleeding, and incipient necrosis at 8 hr, rapidly progressing to total necrosis within 24-48 hr.

Subsequently we studied the effect of PMN depletion in genetically C5 deficient B10.D2 old recipients. The results

of these experiments are also given in Table IV (groups 3-5). In recipients with normal PMN counts the sensitivity of PVG/c skin grafts to 1.0 or 2.0 ml of MARG was similar to that of the grafts in COVF treated C57Bl/10 mice. PMN depletion did not alter the fate of the grafts after injection of the same doses of antibody, except for some delay in the progression to necrosis (group 3 and 4). However, when PMN-depleted B10.D2 old mice also received COVF treatment, the AAR was mitigated since only 8 of 10 grafts were slowly rejected between days 12 and 14 after transplantation (group 5). In this latter group the macroscopic reaction of the grafts was similar to that in the COVF-treated C57Bl/10 mice that received the same dose of 2.0 ml of MARG (group 2), i.e. only slight edema and hyperemia in the first 24 hr after injection, with cyanosis in the following days and a slow progression to complete necrosis. Histologically the picture in the PMN-depleted B10.D2 old mice that had not received COVF treatment was characterized by vasodilation of the graft vessels in the first 2 hr, followed by platelet aggregation and bleeding at 8 hr, and partial necrosis at 24 hr, leading to complete rejection of all grafts at day 13 after transplantation

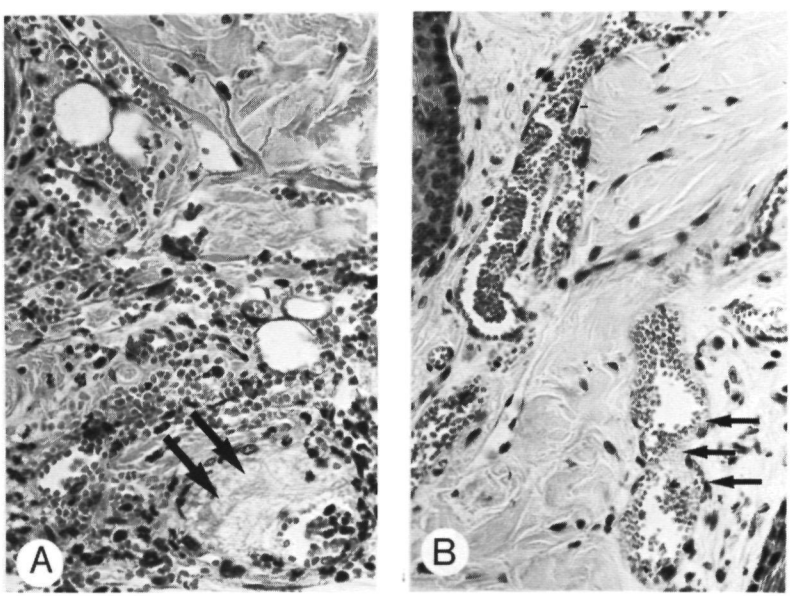

Figure 2. Reaction in the vessels of PVG/c skin grafted onto COVF-treated B10.D2 old mice, at 8 hr after injection of 2.0ml MARG. A: The grafts in recipients with normal PMN counts show extensive vessel wall damage, with PMN infiltration, intravascular platelet aggregation (arrows), and bleeding (H&E, 100x). B: In PMN-depleted recipients the reaction is less severe, but nevertheless vasodilation is present with intravascular platelet aggregation (arrows) and partial disappearance of endothelial cells. (H&E 80x).

(groups 3 and 4). In PMN-depleted B10.D2 old mice that also received COVF treatment (group 5) the microscopic events were comparable to those in similarly treated C57Bl/10 mice (group 2), i.e. moderate dilation and hyperemia of the vessels at 8 hr with local platelet aggregation (Fig. 2B), slowly proceeding to necrosis after several days. Control mice, treated with COVF but not PMN-depleted, showed at 8 hr extensive PMN infiltration in addition to platelet aggregation and bleeding (Fig. 2A).

In the complement normal groups as well as in the complement depleted and complement deficient groups, virtually no PMNs could be found in the sections in the first 8 hr after injection of MARG, neither in the graft vessels nor in the recipient tissue of the PMN-depleted animals. At later stages, from 24 hr, only very few PMNs were present, located in the interstitial tissue along the borderline between graft and host, apparently recruited to this area from the few numbers in the circulation. Migration of these PMNs into the graft was seen only at the time of incipient necrosis. By this time their number had gradually increased but was still very low and only a small fraction of the large numbers of PMNs that could be seen from 2 hr in the non-irradiated control groups.

DISCUSSION

In antibody-mediated rejection of skin and whole organ grafts the major histocompatibility antigens on the endothelial cells are the primary target for the immunologic attack. We have not analyzed the distinct specificities of antibodies present in our antirat serum, because there is sufficient data that xenogeneic sera prepared in a similar way in several species contain antibodies against these antigens. Indeed, it was with a xenogeneic rabbit antimouse serum that the H2-complex of the mouse was first discovered (20). More recent evidence can be derived from a report that rabbit antirat sera detect rat allo-antigens (21), and from the finding that mouse monoclonal antibodies against rat allo-antigens can be raised after immunization with rat erythrocyte or splenic antigens (22).

Studies on the role of PMNs in the AAR have resulted in conflicting reports in the literature. Forbes et al (9) found that AAR in cardiac allografts could be elicited in the absence of PMNs, whereas Wartenberg et al (10) induced an in vitro "rejection" of rabbit and rat hearts by perfusion with allogeneic antisera that contained complement but no

lymphocytes or PMNs. On the other hand Winn et al (8) reported that PMN depletion inhibited the AAR of rat skin grafted onto mice, in a model similar to that in the present study. The differences in results may be partly related to the methods used to effect PMN depletion in the recipients of the grafts. In most experiments this depletion of PMNs is induced by administration of antigranulocyte serum and/or cytostatic drugs. Apart from the variability in the effected PMN depletion the use of antigranulocyte serum may also induce a considerable decrease in complement levels in the recipients, which can be critical, especially when the dose of antiserum that is injected to induce AAR is only sufficient to induce an Arthus-like response in the graft (7). Another consequence of the use of antigranulocyte serum and cytostatic drugs is the adverse effect of these treatments on the condition of the animals. Since a bad condition of the graft recipients leads to impairment of the peripheral circulation, one cannot be certain that the injected antibody will reach the graft vessels in amounts that are representative for the injected dose. There is evidence both from experimental and clinical studies that an impaired blood flow to the graft can protect it from rejection by antibodies (23). When a model is used in which the grafts are primarily vascularized by direct connection of the graft vessels to large arteries of the recipient such as the aorta (9) there is no great risk of an important impairment of the blood flow in the graft, due to deterioration of condition of the recipient. However, in skin graft models the effect of a suboptimal condition on the circulation of the graft is likely to be critical. By using TBI for induction of PMN depletion we could keep the mice in good condition. The results given here were all obtained in groups of healthy active mice with warm good looking skins. The only situation in which we were not able to keep the mice healthy were the groups that in addition to TBI received COVF treatment. Although daily injection of COVF did not visibly harm the control mice, the irradiated mice reacted with increasing deterioration of condition, gradual loss of activity and coldness of the skin. This reaction was reversible within 1-2 days after discontinuation of the COVF treatment. It may be that a temporal decrease in the peripheral circulation was responsible for the delay and partial inhibition of the reaction to the MARG in these groups (Table IV, groups 1, 2, 5). Therefore we feel that from the differences in reaction to high doses of MARG between COVF treated C57Bl/10 mice and the B10.D2 old mice that did not receive COVF treatment no conclusions can be drawn with regard to possible differences in the action of the early and late complement components.

Our results show that although PMNs play a role as amplifiers and accelerators of tissue necrosis induced by acute antibody-mediated vasculitis, they are not essential mediators in the basic damage to the endothelial cells, initiated by the

binding of antibody to the antigens located on the cell membrane. The outcome of the experiments in irradiated recipients with a normal complement system that received high doses of antibody was not surprising, as we have shown earlier that in these cases a Shwartzman-like type of rejection can be seen, characterized by early platelet aggregation without preceding accumulation of PMNs in the graft vessels (6). However, for the acute rejection developing after administration of low doses of antibody we expected PMNs to be essential, as in this type of vasculitis destruction of the endothelial cells was preceded by accumulation of PMNs in the vessels (6). The appearance of PMNs was even more impressive in complement depleted mice, wherein large doses of antibody were necessary for the induction of AAR (7). The results of the present study prove that our previously held hypothesis of an essential role for PMNs as mediators in the endothelial destruction in the Arthus-like type of rejection is no longer tenable. In complement normal recipients one can assume, as has been suggested by others (24-26), that the binding of antibody to the endothelial cell membrane may be followed by a complement induced cytolysis. If this assumption is right, the finding that PMNs are not essential mediators implies that the morphologic differences between the Shwartzman-like and the Arthus-like types of AAR may be mainly a consequence of a difference in the velocity of the lytic reaction. When the total damaging potential of antibody and available complement is sufficient to cause immediate endothelial cell lysis, the thrombogenic subendothelial structures will cause early platelet aggregation. The resulting vasculitis will thus resemble the generalized Shwartzman reaction, in which the endothelial cells are destroyed by bacterial endotoxins. On the other hand, if the amounts of antibody and/or complement are only sufficient to effect a moderate, more slowly developing damage to the endothelial cells, the process of cell lysis and subsequent platelet aggregation will take several hours. In this situation platelet aggregation is microscopically not visible behore 8 hr after injection of the antibody. Meanwhile the chemotactically attracted PMNs dominate the morphological picture and as the initial partial endothelial damage is not easy to detect, this sequence of events simulates that of an Arthus reaction.

The assumption of complement induced cytolysis as the basic event in the AAR does not explain the occurrence of AAR in COVF treated or C5 deficient recipients. One could argue that in COVF treated mice unmeasurable traces of complement could still be operative. However, it is generally assumed that C5 deficient mice completely lack the ability to form the lytic complex of complement. We also were unable to detect measurable levels of hemolytic complement activity in this strain (17). Moreover, the finding of Perden et al (15) that AAR could be induced by injection of purified non-complement-fixing IgG1 subclass antibodies, strongly

suggests that a complement independent mechanism can be effective in the AAR. Since our results show that PMNs cannot be hold responsible for the complement-independent cell damage, one has to consider other mechanisms by which large amounts of antibody can induce cell destruction. The recent study of Soper et al (27), who reported lysis of antibody-coated cells by platelets in the presence of early complement components, has focussed new attention on a possible cytolytic effector role for platelets. We cannot exclude that cytolysis by platelets is responsible for the endothelial cell damage in our experiments with complement-normal and C5 deficient mice, but it is hard to see how this can occur in COVF treated mice, which lack the early components that are necessary for platelet induced lysis. Another destructive mechanism that could possibly be involved in the neutrophil and complement independent endothelial cell damage is antibody-dependent cell-mediated cytoxicity (ADCC), in which mononuclear effector cells such as macrophages can adhere to antibody-bearing cells by means of their Fc receptors. There is evidence that this phenomenon, which has mainly been described in in vitro systems, can also be effective in vivo and this activity is not inhibited by Cobra Venom Factor treatment (28). In the histologic sections we could find no indication that mononuclear cells played a role in the initiation of the vasculitis. Also other experimental data suggestive for a destructive role for macrophages in the initial phase of antibody-induced immune vasculitis are lacking. However, this does not exclude a possible effector role for these cells, and it is conceivable that radioresistent monocytes, macrophages, or other Fc-receptor bearing effector cells induce the cell damage.

We now have evidence that also allogeneic mouse skin grafts can be destroyed by specific alloantibody in the absence of PMNs (29). This raises the question whether this PMN-independent destructive mechanism can be operative in other forms of antibody-mediated vasculitis and therefore also in the classical Arthus reaction. It is generally held that in the Arthus reaction damage to the endothelial cells is caused by the release of lysosomal enzymes from PMNs that have been attracted by chemotactic action of activated complement factors. However, there is some evidence that PMNs are not completely indispensable. Cochrane has found that the Arthus reaction can take place in the absence of PMNs if very high doses of antibody are injected (1). Also in a related Arthus-type reaction, evoked by intradermal injection of high doses of antibody to basement membrane, vessel wall damage could be induced in the absence of both complement and PMNs (1). Furthermore, the occurrence of complement and neutrophil independent glomerular injury after injection of high doses of antibody against glomerular basement membrane has been fairly well established in several models of experimental nephritis (19, 30), which share many similarities with both the Arthus

reaction and certain types of human glomerulonephritis. The pathogenesis of these types of complement and PMN-independent injury is thus far unknown.

In conclusion, our results show that in the AAR PMNs are not essentially involved in the initial attack on the endothelial cells, caused by the binding of the antibody to the antigen on the cell membrane. This implies that for the basic damage to the endothelial cell membrane other factors are responsible. In complement normal recipients this may be a direct lytic effect induced by the activation of complement. However, the mechanism of endothelial cell damage that can be elicited by high doses of antibody in the absence of complement and PMNs remains to be clarified.

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CHAPTER 6

POLYMORPHONUCLEAR GRANULOCYTES ARE NOT ESSENTIAL FOR THE ACUTE ANTIBODY-MEDIATED REJECTION OF MURINE SKIN ALLOGRAFTS

M. José J.T. Bogman, Ine M.H.A. Cornelissen,
and Robert A.P. Koene

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ABSTRACT

The role of polymorphonuclear granulocytes (PMNs) in the acute antibody mediated rejection (AAR) was studied in a murine skin allograft model. B10.A skin was grafted onto B10.D2 recipients that were treated with goat anti-mouse lymphocyte serum, to postpone cellular rejection. This prolonged the median survival time of the grafts to 21.5 $\pm$ 1.0 days. PMN depletion was effected by a Total Body Irradiation of 6.0 Gy on day 4 after grafting, which reduced the number of PMNs in the circulation to levels below 150/µl from day 3 to 10 after the irradiation. To induce AAR the mice received, on day 8 after grafting, an i.v. injection of a monoclonal antibody with specificity against the donor H-2K^k antigen together with 0.25ml fresh rabbit serum. This caused acute rejection of all grafts in the control group within 72 hr, with abundant presence of PMNs in the graft vessels on histologic examination. In PMN depleted recipients the reaction in the grafts was somewhat retarded in the first 24 hr, but nevertheless acute rejection occurred within 72 hr in 9 of 11 animals, whereas PMNs were virtually absent from the grafts. The results show that PMNs act as accelerators and amplifiers of the acute antibody mediated rejection process, but are not essential mediators in the damage to the vascular endothelium of the grafts.

In hyperacute antibody-mediated rejection of human kidney allografts accumulation of polymorphonuclear granulocytes (PMNs) is an early event in the vasculitis that eventually leads to destruction of the graft. Analogies have been drawn to the Arthus reaction (1-3) in which PMNs are also characteristically present in the early hours after the binding of antibody and antigen. Since in the Arthus reaction PMNs are generally held to be essential mediators in the generation of the ultimate tissue damage, several experimental models have been employed to study the role of PMNs in the induction of acute antibody mediated graft rejection (AAR) (4-7). However, the results of these studies have been conflicting (4,6,7) and there are still controversial opinions about the role of PMNs in the initial attack on the endothelial cells of the graft, that starts with the binding of the antibody to the cell membrane.

In previous studies of our group it was shown that in immunosuppressed mice that carried skin allografts, hyperacute rejection can be induced by intravenous injection of specific antibody, directed against the graft tissue, together with an efficient exogenous complement (5, 8). Morphologically, the first reaction after injection of antibody and complement was edema, dilatation of the graft vessels, and accumulation of PMNs in the vessels, present at 30 min and maximal at 2-4 hr. The early presence of PMNs led to the hypothesis that these cells were attracted by chemotactic factors generated by the binding of antibody to antigen and the activation of complement, and that they were essential or at least very important effectors in the damage to the endothelial cells, by means of their release of lysosomal enzymes during phagocytosis of the immune complexes. The aim of the present study was to test the influence of PMN depletion on antibody-mediated rejection of skin allografts.

MATERIALS AND METHODS

Animals. Inbred strains of B10.D2/new Sn($H2^d$) and B10.A ($H\text{-}2^k$) mice, originally obtained from the Jackson Laboratory (Bar Harbor, Maine), were kept in our laboratory by continuous brother-sister matings. A goat, used to prepare anti-mouse lymphocyte serum, was bought on the local market. Fresh frozen serum of New Zealand white rabbits was used as a source of complement.

Antisera: Goat anti-mouse lymphocyte serum (GAMLS) was prepared as described earlier (9). Cells that produce the monoclonal antibody coded 11-4.1, which recognizes the specificity $H\text{-}2K^k$ (10), were kindly provided by the Salk Institute, San Diego, CA. The antiserum 11-4.1 was produced in ascites fluid using BALB/c mice. The cytotoxic titer, determined in a trypan blue exclusion assay using B10.A spleen cells, was $1:5x10^4$.

Transplantation procedure. Squares of 0.8 x 0.8 cm full-thickness skin of female B10.A mice were grafted onto the flanks of 3-4 months old female B10.D2 recipients by a modified fitted graft technique (8). The fate of the grafts was followed by daily macroscopic inspection. The grafts were considered to be rejected when no viable epithelium remained. Median survival times (MST) and standard errors were calculated according to the method of Litchfield (11). To postpone cellular rejection of the grafts the recipients were treated with GAMLS on days -2, 0, 2, and 4 after grafting in a dose of 0.25 ml i.p. The MST of the grafts under this regimen was 21.5 $\pm$ 1.0 days. In the mice that received TBI on day 4 after transplantation the injection of GAMLS on day 4 was omitted. Under these conditions the MST of the grafts was 23.3 $\pm$ 1.2 days.

PMN depletion. Mice were depleted of PMNs by Total Body Irradiation (TBI) in a single dose of 6.0 Gy (600 rad), on day 4 after transplantation, at a dose rate of 0.20 Gy min^{-1}, from a 250 kV X Ray source (Stabilipan, Siemens). To undergo TBI the mice were, after 24 hr of fasting, anaesthetized by an i.p. injection of sodium pentobarbital, 0.03 mg per g body weight (Narcovet, Apharma BV, Arnhem, The Netherlands) and placed between two perspex plates, the upper plate being 2 mm thick. The grafts were covered with a small piece of lead (12x12x3 mm) to prevent irradiation damage to the endothelial cells of the graft. The side scatter dose on the graft tissue, as calculated in a phantom set up was 20-25% of the total dose of 6.0 Gy. From two days before until 3 weeks after TBI the mice were kept under aseptic conditions in a laminar flow unit, and received

Polymyxin B (40 mg/l) and Neomycin (100 mg/l) in their drinking water, as well as heat sterilized food. Under this regimen the irradiated mice remained in good condition.

Acute rejection studies. To induce acute antibody mediated rejection (AAR) the mice received, at day 8 after transplantation, an i.v. injection of the monoclonal antibody 11-4.1 in a dose of 3200 cytotoxic units (CTU), together with 0.25 ml fresh rabbit serum (RC). One CTU was defined as the amount of alloantibody lysing 50% of 1.25×10^5 B10.A cells in a cytotoxic trypan blue exclusion assay. AAR was defined as macroscopic necrosis of more than 80% of the graft tissue within 72 hr after the injection of antibody and RC.

Histologic examination of the graft. For histologic examination representative grafts and surrounding recipient tissue of the PMN-depleted and the control mice were excised at point 8, 24, and 72 hr after injection of antibody and RC. After fixation in buffered formalin and embedding in paraffin, 4µ sections were cut and stained with hematoxylin and eosin (H&E), Giemsa, Goldner's trichrome stain, and Mallory's Phosphotungstic acid hematoxylin stain.

RESULTS

PMN depletion. TBI of 6.0 Gy invariably caused a rapid fall of PMNs in the peripheral blood of the mice to values lower than 150/µl (in 90% of the animals even lower than 100/µl) and these values remained at this low level from day 3 to 10 after irradiation (in preparation). The effect of the TBI in the present experiment was monitored by counting PMNs in blood drawn from the retrobulbar plexus of two mice of the irradiated and the control group at day 8 (0 hr and 8 hr after the injection of antibody and RC) and of three mice of each group at day 11 (72 hr after injection). Mean numbers of PMNs in the mice of the irradiated group were 41/µl (0 hr) and 25/µl (8 hr) at day 8, and 22/µl at day 11 after transplantation. In the control group the corresponding numbers were 1153, 2948, and 676. Platelet numbers were also reduced by TBI but in the first 7 days after the irradiation, i.e. the period in which AAR developed, the counts always remained above 50% of normal values.

Acute rejection. AAR was induced on day 8 when the grafts were in excellent condition and showed no signs of rejection. The effects of an i.v. injection of 3200 CTU of monoclonal antibody 11-4.1 combined with 0.25 ml RC are shown in Table I.

Table I. The influence of PMN depletion on the occurrence of AAR in B10.A skin grafted onto immunosuppressed B10.D2 recipients

Treatment[1]	PMN Depletion[2]	Acute Antibody Mediated Rejection[3]
+	-	9/9
+	+	9/11
-	-	0/10 (MST 21.5 ± 1.0 days)
-	+	0/10 (MST 23.3 ± 1.2 days)

1) Specific anti-H2-K^k monoclonal antibody was injected i.v. on day 8 after transplantation, in a dose of 3200 CTU, together with 0.25 ml fresh rabbit serum (RC).
2) PMN depletion was induced by TBI of 6.0 Gy on day 4 after transplantation.
3) Number of mice with necrosis of more than 80% of the graft tissue within 72 hr after injection of antiserum and RC/number of mice tested.

The results demonstrate that AAR could not only be induced in control mice but also in PMN-depleted recipients. Macroscopically the reaction of the grafts in both groups was essentially the same, although in the PMN-depleted group the development of the ultimate necrosis was somewhat retarded. At point 2 hr after injection all grafts showed edema and some red-blue discoloration, the reaction in the PMN-depleted group being slightly less intense. At 8 hr most of the grafts in the control group showed cyanosis and part of them patchy bleeding. In the PMN-depleted group cyanosis occurred in about 50% of the grafts, and only few had signs of bleeding. At 48 hr all grafts of the control group were completely necrotic, whereas in the PMN-depleted group this was the case in 3 out of 11. However, at 72 hr 9 out of 11 of the PMN-depleted animals had total to subtotal (80% or more) necrosis of their grafts, proceeding to rejection. The two remaining grafts partly recovered from the initial cyanosis and bleeding, and were eventually rejected at days 22 and 24 after transplantation.

Graft histology. Microscopic examination of the grafts in the control group showed, at 8 hr after injection of antibody and RC, marked dilation and congestion of the vessels, accompanied by vessel wall destruction, platelet aggregation, and bleeding (fig. 1A). Many PMNs were present in the vessels and in the

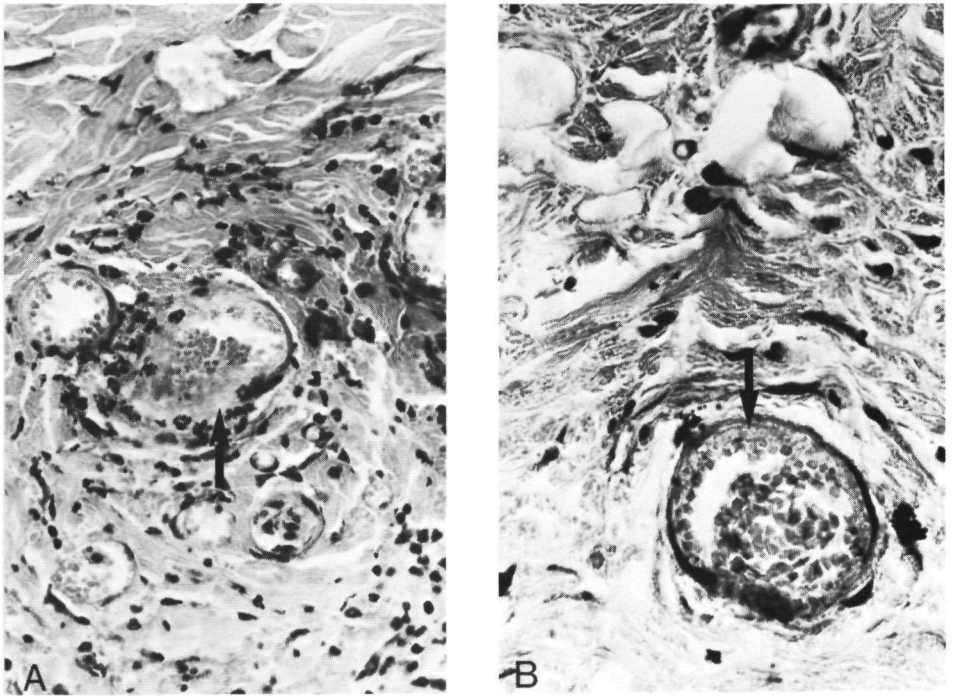

Figure 1. Vascular reaction in the grafts at 8 hr after injection of antibody and complement. A. The control group shows many PMNs in and around the vessels, severe vascular damage, and platelet aggregation (arrow). (Giemsa 100x). B. In the PMN depleted group no PMNs are seen. Nevertheless endothelial damage and platelet aggregation (arrow) are evident. (Giemsa 160x).

interstitial tissue of both graft and recipient. At 24 hr the vessels were completely destroyed and there was subtotal necrosis, accompanied by heavy infiltration of PMNs especially along the graft border. In the PMN-depleted group at 8 hr a less extensive but nevertheless obvious reaction was seen in the graft vessels, consisting of vasodilation, local vessel wall damage, platelet aggregation (fig. 1B), and local bleeding. In these sections no PMNs could be found in the vessels. At 24 hr all changes were more pronounced, and there was partial necrosis of the graft. Also at this time PMNs were absent from the grafts and could only be found sporadically along the graft border, apparently recruited to this area from the small numbers left in the circulation after the TBI. The macroscopic diagnosis of rejection could be confirmed histologically in all grafts taken at 72 hr.

Our results show that in the AAR of mouse skin allografts, induced by intravenous injection of specific antibody and complement, PMNs are not essential for the induction of the initial endothelial damage, although these cells have an accelerating and amplifying effect in the secondary events that lead to the ultimate graft destruction. This finding is consistent with data in the literature reporting the occurrence of AAR in cardiac allografts in rats in the absence of PMNs (6), and the induction of an in vitro "rejection" of rabbit and rat hearts by perfusion with allogeneic antisera containing complement but no lymphocytes or granulocytes (7). On the other hand it was found that in mice carrying rat skin xenografts PMN depletion prevented the AAR induced by injection of antirat globulin (4). Considering the controversial conclusions from studies on different models we originally assumed that these could be explained by the fact that the AAR of allografts and xenografts can take place along different patterns, i.e. either along an Arthus-like or along a Shwartzman-like type of vasculitis (2-4, 12). As the Arthus type of AAR is characterized by early PMN accumulation in the vessels of the graft and the Shwartzman type by early intravascular platelet aggregation, the pathogenesis of the initial endothelial damage may be essentially different in these two types. PMNs may be essential mediators in the Arthus-like AAR, whereas platelets may play an important role in the Shwartzman-like type. However, in a rat to mouse skin xenograft model, in which we were able to change an Arthus-like pattern of AAR into a Shwartzman-like one by simply modifying the amounts of injected antibody and available complement (9, 13), we could not prevent the occurrence of AAR in either type by depleting the recipient mice of PMNs (in preparation). The results in the present report are consistent with these findings in the xenograft model and are in favor of the hypothesis held by several authors (3, 7, 14) that the initial damage to the graft endothelium is caused by a direct cytolytic effect of the binding of antibody to the antigen on the cell membrane and the subsequent activation of complement. Platelet aggregation to the damaged vessel walls may subsequently amplify the process. Furthermore, there is recent evidence that mouse platelets can have a direct lytic effect on antibody coated cells if early complement components are present (15). Such a hypothesis is only valid for those conditions wherein an efficient complement is available. In our allograft model this can be effected by injection of a heterologous complement together with the antibody (5). However, the AAR of rat xenografts in mice does not require such an exogenous complement source. We have previously shown that in this model AAR can also be induced in recipients that are congenitally complement deficient or complement depleted by treatment with Cobra Venom Factor (9, 16). Under these

conditions the amount of antibody, necessary to induce AAR is substantially higher than in complement-normal recipients, but nevertheless AAR does take place, even in the absence of PMNs (in preparation). Our present data give no further insight in these PMN- and complement-independent mechanisms.

In conclusion we have shown that PMNs are not essential mediators in acute antibody-mediated rejection of mouse skin allografts, although they can amplify and accelerate the reaction. It seems likely that the basic damage to the endothelial cells is brought about either by direct complement mediated cytolysis of endothelial cells with consequent platelet aggregation and vascular obstruction or by platelet mediated cytolysis of endothelial cells.

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CHAPTER 7

VARIABLE EXPRESSION OF Ia ANTIGENS ON THE VASCULAR ENDOTHELIUM OF MOUSE SKIN ALLOGRAFTS

R.M.W. de Waal, M.J.J.T. Bogman, C.N. Maass, L.M.H. Cornelissen, W.J.M. Tax, and R.A.P. Koene

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Variable expression of Ia antigens on the vascular endothelium of mouse skin allografts

R. M. W. de Waal, M. J. J. Bogman, C. N. Maass, L. M. H. Cornelissen, W. J. M. Tax & R. A. P. Koene

Department of Pathology and Department of Medicine, Division of Nephrology, Sint Radboudziekenhuis, University of Nijmegen, Nijmegen, The Netherlands

Ia antigens are membrane-bound glycoproteins that play a part in antigen recognition and subsequent cell–cell interactions in the immune response. In the mouse they are coded for by the *I* region of the major histocompatibility complex *H-2* and have been demonstrated on B lymphocytes, monocytes, activated T cells, macrophages and dendritic cells, including Langerhans cells[1,2]. Ia-like antigens have also been detected on the vascular endothelium in man[3–6] and on epidermal keratinocytes in rats[7,8] but expression on the latter cells was induced by a graft-versus-host reaction or by contact hypersensitivity[9]. In the mouse, previous studies have suggested that Ia antigens in skin are restricted to epidermal Langerhans cells and it was thought that these were the targets for Ia-dependent rejection of skin allografts. The results presented here show that Ia antigens in mouse allografts are also present on the vascular endothelium but their expression is variable and dependent on the immunological status of the recipient. These findings suggest that vascular endothelial cells can act as targets in Ia-incompatible skin allograft rejection.

We investigated the presence of Ia antigens in mouse skin allografts and the localization of these antigens by *in vivo* injection of labelled anti-Ia alloantibodies. A monoclonal anti-Ia IgG2a alloantibody, raised in our laboratory in BALB/c ($H\text{-}2^d$) mice against BALB/K ($H\text{-}2^k$) donor cells, was used. The Ia^k determinant recognized by this antibody was shown to map in the *I* region by a positive binding reaction with 40–50% of B10.AQR (Ia^k) spleen lymphocytes and a negative reaction with B10.T (6R) (Ia^q) cells. These strains differ only at the *I* and *S* region of the *H-2* complex. The anti-Ia antibody was labelled with ^{125}I using the chloramine-T method[10] and aliquots of approximately 5×10^4 c.p.m. were injected intravenously (i.v.) into otherwise untreated female B10.D2 ($H\text{-}2^d$) recipients of B10.BR ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) tail skin allografts on day 6 after transplantation. The grafts were excised after different time intervals and the amount of radioactivity

was determined by γ-counting of the entire graft.

Figure 1 shows that specific binding of the anti-Ia antibodies to the B10.BR grafts occurred, with a maximum at 8–12 h after injection. In the BALB/c control grafts a background binding level was observed after each time interval. To localize the bound radioactive antibodies, B10.BR skin grafts were excised 16 h after injection and prepared for autoradiography (see the legend to Fig. 2). Microscopic examination showed that the bound radioisotope was localized in the vessel walls of the donor skin (Fig. 2*a*). The vessels of the recipient, visible in a rim of tissue surrounding the graft, were negative in all cases. All other structures of donor origin, including the epidermis, were also negative. To identify the localization of the vessel wall associated Ia antigens more precisely, the monoclonal anti-Ia was labelled with biotin and the conjugate was administered as outlined above. After excision of the grafts cryosections were prepared and developed with avidin conjugated with fluorescein. The capillary endothelial cells were then identified in the same sections by incubation with rabbit antiserum against factor VIII antigen, which is a specific marker for endothelial cells[11,12], followed with rhodamine-conjugated goat anti-rabbit IgG serum. Both labels completely overlapped, which showed that the cells that bind the anti-Ia antibodies are

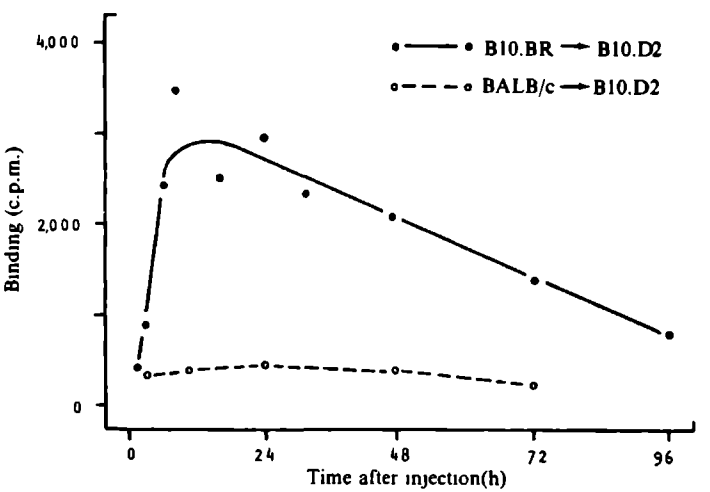

Fig. 1 Binding of ^{125}I-labelled anti-Ia^{k} monoclonal IgG2a to B10.BR (●– –●) and BALB/c (○– –○) skin allografts *in vivo*. Female B10.D2 recipients were injected intravenously on day 6 after transplantation with 0.1 ml phosphate-buffered saline containing 0.1% bovine albumin and 3.8×10^4 c.p.m. of radiolabel. After different time intervals the recipients were killed, the grafts excised, and the amount of radioactivity determined. Each point represents the mean value of two measurements.

cells of the vascular endothelium (Fig. 2*b*, *c*). In control skin allografts from BALB/c ($H\text{-}2^d$) donor mice, binding of biotinylated anti-Ia^k antibodies was undetectable. The binding of a control monoclonal antibody (WT3, raised against human T lymphocytes) that was injected into B10 . D2 recipient mice carrying B10 . BR allografts was also negative.

In these experiments we had observed a correlation between the binding of labelled anti-Ia antibodies and the degree of rejection. Grafts showing macroscopic signs of rejection bound considerably more antibody than healthy grafts. Perhaps expression of donor strain Ia antigens on the vascular endothelium of the allograft might be influenced by the immune response of the host. To investigate this, donor tail skin was transplanted onto immunosuppressed and nude recipients. *In vivo* binding studies were performed at day 7 after transplantation by i.v. injection of radiolabelled monoclonal anti-Ia antibody followed by excision and γ-counting of the skin grafts 16 h after injection. The results of these experiments are shown in Table 1. A high binding of anti-Ia antibodies was observed if donor skin from mice carrying Ia^k antigens (B10 . BR or B10 . AQR) was grafted onto untreated normal recipients. Background levels of binding were seen if the donor skin lacked the Ia^k antigens (B10 . D2), even in the presence of an *H-2* incompatibility between donor and recipient (B10 . T (6R) → B10 . D2) Similar results were obtained with the monoclonal anti-I-E/C^k antibody coded 14-4-4 which was prepared by Ozato *et al.*[13] (data not shown). In recipients treated with immunosuppressive agents such as rabbit anti-mouse lymphocyte serum or cyclosporin A (Cy A), there was also only background binding of radioactivity in skin grafts of Ia^k-positive donors, demonstrating the dependence of endothelial Ia antigen expression on the immune response of the host (Table 1). A binding inhibition assay showed that the anti-mouse lymphocyte serum did not contain anti-Ia xenoantibodies that could interfere with the *in vivo* binding studies (data not shown). If congenitally athymic BALB/c *nu/nu* mice, which lack a T-dependent immune system, were used as recipients of B10 . BR grafts, binding of the radiolabelled anti-Ia to the donor tissue did not occur either, suggesting that the lack of Ia-binding is due to the absence of an immune response and not to a direct interaction between the immunosuppressive drugs and the endothelial cells. The absence of binding of anti-Ia in these allografts was confirmed by immunofluorescence; on day 62 after transplantation on nude recipients, no positive fluorescence staining was observed on donor endothelial cells.

We have also examined anti-Ia binding after withdrawal of immunosuppressive treatment. In this experiment, B10 . T(6R) recipients of B10 . AQR skin grafts received daily Cy A and the uptake of monoclonal Ia antibody by the graft was assessed

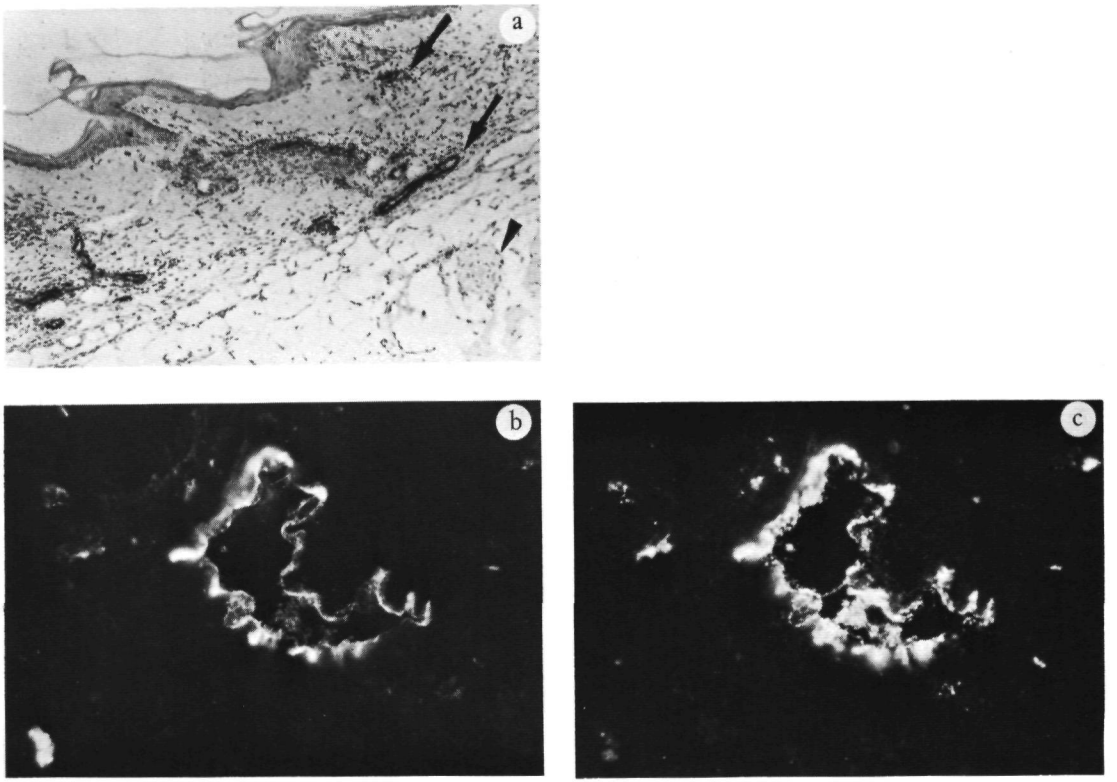

Fig. 2 ***a***, Autoradiomicrograph showing the localization of ^{125}iodine-labelled monoclonal anti-Ia IgG2 in B10.BR skin allografts after passive administration to BALB/c recipients *in vivo*. ×31. Male recipient mice were injected intravenously at day 6 after transplantation with 0.1 ml phosphate-buffered saline (PBS) containing 0.1% bovine albumin and 17×10^4 c.p.m. of ^{125}I-labelled anti-Ia^k IgG2. Grafts were removed after 16 h and put in neutralized 4% formaldehyde solution. After fixation and imbedding, 5-μm paraffin sections were cut and transferred to gelatinized slides. The sections were deparaffinated, rinsed in distilled water and dipped in photographic emulsion (Nuclear Research Emulsion, Ilford K5, 3 × diluted with water) in the dark. The slides were air-dried overnight in a humid atmosphere at room temperature and then left for 4 weeks in sealed boxes at 4 °C. After exposure the emulsion was developed with developing fluid (Kodak), and the sections were stained with haematoxylin and eosin. Photomicrographs were made with black and white film (Agfapan-25) using a green filter. The localization of the intravenously injected anti-Ia antibody is clearly visible as a positive staining of the vessel walls of the graft (arrows). The vessels of the recipient are negative (arrowhead). ***b***, Micrograph showing the localization of biotin-labelled anti-Ia^k in a B10.BR skin allograft after passive administration to a B10.D2 recipient *in vivo*. The figure shows the presence of fluorescein isothiocyanate-labelled anti-Ia antibodies on the endothelium of the graft, demonstrating the presence of donor Ia^k antigens. ×78. 1 mg of monoclonal IgG2a was conjugated with 120 μg of the *N*-hydroxysuccinimide ester of biotin (Sigma) in 1 ml of 0.1 M $NaHCO_3$ at room temperature. 100 μl of the conjugate were injected intravenously on day 6 after transplantation of both a B10.BR and a BALB/c graft onto an untreated B10.D2 recipient. After 2 h the grafts were excised and frozen in liquid nitrogen. Cryosections 4–5-μm thick were air dried, fixed in acetone for 10 min and washed with PBS. The sections were then incubated in avidin-FITC (Sigma), diluted 500× in PBS containing 0.1% bovine serum albumin (BSA), and washed again. Subsequently the tissue sections were incubated with rabbit antiserum against human factor VIII antigen (Red Cross, Amsterdam) diluted 100×, washed and developed with goat antiserum against rabbit IgG, conjugated with rhodamine (Cappel), diluted 50×. Finally the sections were rinsed, mounted in Aquamount (Gurr) and examined under a Leitz Dialux fluorescence microscope, equipped with Ploemopak epi-illuminator and filter combinations for fluorescein and rhodamine labels. ***c***, Using the filters for rhodamine fluorescence, the binding of the anti-factor VIII antibodies is shown in the same section as in *b* demonstrating the presence of factor VIII antigen on the endothelium. ×78.

at different days after transplantation. In five mice the Cy A administration was discontinued on day 14. This leads to rejection of the skin grafts within 10–13 days[14]. The results show that withdrawal of the immunosuppressive agent resulted in uptake of radiolabel within 3 days. In the group that received Cy A throughout, binding was not detectable at any time (Fig. 3).

Our results show that donor type Ia antigens are present on vascular endothelium of skin allografts in the mouse. Their expression increases at the onset of rejection, whereas T-cell deficiency or administration of immunosuppressive agents suppresses it. Subsequent withdrawal of immunosuppression

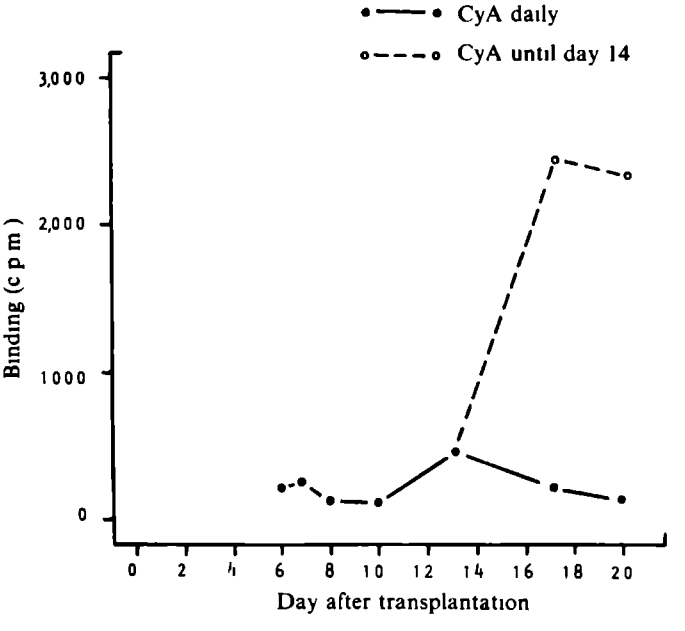

Fig. 3 Binding of ^{125}I-labelled anti Ia^k monoclonal IgG2a to B10 AQR skin allografts *in vivo* Sixteen female B10 T(6R) recipients of B10 AQR grafts daily received 75 mg kg^{-1} cyclosporin A (Sandoz) orally, dissolved in olive oil at a concentration of 20 mg ml^{-1} After different time intervals binding of radiolabelled anti-Ia^k was determined For this, recipient mice were injected intravenously with $4\,0 \times 10^4$ c p.m and killed 16 h later, to determine the amount of binding in the allograft At day 14 after transplantation the remaining 8 mice were divided in two groups, one in which the immunosuppression was continued and one in which the administration of cyclosporin A was stopped Most of the points represent the mean value of 2–3 measurements

Table 1 Binding of radioiodinated anti-Ia^k monoclonal antibodies to mouse skin allografts at day 7 after transplantation

Donor	Recipient	H-2 haplotype	No. mice	Treatment	Binding ± s.d. (c.p.m.)
B10.BR	BALB/c	k→d	5	—	2,900 ± 750
B10.D2	BALB/c	d→d	5	—	350 ± 100
B10.T(6R)	B10.D2	y2→d	6	—	510 ± 80
B10.BR	B10.D2	k→d	10	—	2,500 ± 600
B10.BR	B10.D2	k→d	10	RAMLS*	330 ± 90
B10.AQR	B10.T(6R)	y1→y2	6	—	2,038 ± 650
B10.AQR	B10.T(6R)	y1→y2	5	Cyclosporin A†	370 ± 10
B10.BR	BALB/c *nu/nu*	k→d	8	—	450 ± 100
B10.D2	BALB/c *nu/nu*	d→d	5	—	390 ± 150

* Rabbit-anti-mouse lymphocyte serum (0.25 ml) was injected intraperitoneally at days 0, 2 and 4 after transplantation.
† Cyclosporin A (Sandoz) was dissolved in olive oil at a concentration of 20 mg ml^{-1} and administered orally (75 mg per kg, daily).

results in reappearance of the Ia antigens. A variable expression of Ia antigens has also been found on mouse peritoneal macrophages[15–17]. Ia expression on these cells is regulated by a lymphokine elaborated by activated T cells[18] which is similar to immune interferon[19].

It seems likely that the expression of Ia antigens on vascular endothelium in our system is also under the influence of activated T cells. This would explain why endothelial Ia antigens have never been found in normal mouse skin apart from on epidermal Langerhans cells. We did not observe binding to cells in the epidermis. It is, however, possible that these cells could not be reached by sufficient amounts of antibody to be detectable by autoradiography. After administration of high doses of antibody, labelled with biotin, we have observed occasional binding to basal keratinocytes.

Our results suggest that, in skin grafting across Ia incompatibilities, where Ia antigens on passenger cells initiate the immune response, the same antigens present on vascular endothelium are the primary targets for the rejection process. This latter assumption is supported by a study in allografts of human skin, which showed that microvascular damage invariably preceded epithelial necrosis[20]. This could explain the contradictory results obtained with skin from bone marrow chimaeras grafted to Ia-incompatible recipients[21]. If syngeneic skin, repopulated with allogeneic *I*-region incompatible passenger cells is transplanted, rejection does not occur. This could be because in these skin grafts allogeneic Ia-positive endothelial cells are not available as targets. On the other hand, allogeneic skin grafts repopulated with cells syngeneic to the recipient are also often not rejected in *I*-region incompatible combinations. Under these circumstances the endothelial Ia antigens will not be expressed, because the syngeneic passenger cells fail to stimulate the immune response of the recipient.

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CHAPTER 8

GENERAL DISCUSSION

Acute antibody-mediated rejection as a model of antibody-induced vasculitis

Although hyperacute rejection, caused by the presence of antibodies against histocompatibility antigens, may occur in clinical transplantation this has become a rare event in the last decade, since it can be prevented by careful crossmatching. It has also become clear that not all antibodies are harmful. The presence of lymphocytotoxic auto-antibodies is even accompanied with excellent graft survival (1). The demonstration of antibodies against HLA-A and B antigens of the donor kidney is considered to be an absolute contra-indication for transplantation because these antibodies are probably always destructive, but the role of allo-antibodies against B cells and especially that of HLA-DR antibodies is less clear. They are problably not involved in hyperacute rejection, but whether they play a role in forms of chronic rejection has not been elucidated. Chronic antibody-mediated rejection still remains a problem and at present most of the late graft failures are due to this form of rejection. Therapeutic approaches mainly concentrate on non-specific inhibiton of the immune reaction of the host by cytostatic therapy and corticosteroids, but these treatments mainly influence the cellular immune response and have only a moderate effect on the chronic production of antibody by the host. Despite the fact that non-specific immunosuppressive treatment schedules have greatly improved the outlook for functional graft survival, the increased risk for the patient to acquire opportunistic infections or to develop malignant disease is a high price to pay. This situation can only be changed by the development of methods that achieve a more specific elimination of the immune defense against the graft. In this perspective further insight in the mechanisms of antibody-mediated vessel wall damage in the graft is clearly important. However, the impact of studies on antibody-mediated rejection goes far beyond that of treatment of graft destruction, because they are of importance for our understanding of other forms of immune vasculitis.

Immune vasculitis, defined as immunologically induced necrotizing vessel wall destruction, is either the hallmark or an accompanying feature of many clinical syndromes. Among these are polyarteritis nodosa, systemic lupus erythematosus (SLE), rheumatoid arthritis, Henoch Schönlein purpura, Wegeners´ disease, and several forms of glomerulonephritis. A great deal of the present knowledge of the pathogenesis of immune vasculitis has been derived from studies in experimental models. The various ways in which experimental types of vasculitis are induced, and the histological and clinical differences in the resulting inflammatory processes, have led to different concepts of the pathogenesis of vessel wall damage, and analogous hypotheses have been developed with regard to the causes of clinical syndromes that histologically resemble the experimental models. However, considering the vast amount of clinical and experimental studies that have concentrated on the mechanisms involved in the different types of vasculitis, and the unmistakable progress that has been made, the questions raised largely outnumber the answers that have been given, and, thus far, surprisingly little is known about the essential factors that in a given individual on a given time, lead to the occurrence of immune vasculitis.

Like in the antibody-mediated graft rejection the study of the pathogenesis of immune vasculitis can focus on three main levels: 1) the antigenic targets, 2) the biologic properties of antibodies or immune complexes, and factors determining their deposition in the vessels, and 3) the role of host factors and mediator systems.

In the vast majority of clinical syndromes associated with vasculitis the antigen is unknown, even in those diseases in which "immune complexes" can be demonstrated in the circulation. (In these cases the term "immune complexes" is used for aggregated immunoglobulins rather than for antigen-antibody complexes). It is obvious that in experimental models of vasculitis the antigen is at least roughly known, since it is either injected or used to prepare the antibodies. Nevertheless, a complex antigen such as glomerular basement membrane material or brush border antigen, evokes a polymorphic antibody response directed against multiple antigenic determinants, and even in these experimental models the exact way how and when the antibody reacts with the antigen is not always clear.

That antibodies are involved in many types of vasculitis has been inferred from the demonstration of immunoglobulins in the vascular lesions. However, the binding of antibody and antigen or the deposition of immune complexes in the vessel walls is not sufficient by itself to cause tissue damage. In the resulting inflammation and necrosis effector systems of the host must be essentially involved. Many of the studies on mediator systems in immune vasculitis have concentrated on the classic candidates for effector roles in immunologically induced tissue damage: the complement

system, polymorphonuclear granulocytes (PMNs), platelets, and the coagulation system. The suggestion that these systems were essentially involved came originally from microscopic studies of the vascular lesions, that often showed a characteristic PMN accumulation or extensive platelet aggregation and intravascular coagulation. Still, the ways in which different mediator systems bring about the ultimate lesion are far from clear, and data from experimental models are controversial.

To illustrate current opinions on pathogenic mechanisms in immune vasculitis we will refer to a restricted number of experimental models and discuss the factors involved in the induction of the vasculitis in these models as compared to our findings in the acute antibody-mediated rejection (AAR), with special emphasis on the action of mediator systems. For this purpose we have chosen 5 basic types of experimental immune vasculitis: the Arthus reaction, acute and chronic experimental serum sickness, the Passive Heymann Nephritis (PHN) and Nephrotoxic Nephritis (NTN).

In the classic Arthus reaction a local necrotizing vasculitis is induced by intradermal injection of antigen in an animal in which specific antibodies are circulating in the blood (2,3). Antigen, antibody, and complement can be demonstrated along the vessel wall by immunofluorescence, and in the electron microscope the reactants appear to be deposited between and beneath the endothelial cells, with extension into the lumen and the adventitial spaces (3). As described in the Introduction, the resulting inflammatory reaction is characterized by early accumulation of PMNs in the vessels, maximal at 2-4 hours after injection of the antigen, in later stages followed by vessel wall damage, intravascular platelet aggregation with coagulation, and necrosis.
Experimental acute serum sickness is induced by i.v. injection of one large dose of a heterologous protein (mostly purified bovine serum albumin, BSA) into a rabbit, or other animal. A generalized vasculitis develops during the period from day 7 to 15 after the injection (4, 5). The occurrence of the acute vascular lesions is closely related to the simultaneous presence of both antibody and antigen in the circulation, and by immunofluorescence antigen, antibody and complement can be demonstrated in the vessels (5,6). The histologic lesions are variable, but consist mainly of PMN infiltration and endothelial proliferation, together with signs of increased vascular permeability (5-7).
In the model of experimental chronic serum sickness the antigen (BSA) is administered for several weeks in constant daily doses (8). This leads, under certain circumstances, to the development of a chronic glomerular disease, clinically accompanied with proteinuria, and morphologically characterized by subepithelial deposits of immunoglobulins,

without apparent cellular reaction (8,9). The glomerular lesion resembles that of the Passive Heymann Nephritis.
In the Passive Heymann Nephritis (PHN) heterologous antibodies, raised against autologous tubular antigens, are injected i.v. into a rat (10,11). This leads to the development of a glomerular lesion, that like that of chronic serum sickness, is characterized by epimembranous, i.e. subepithelial depositions of immunoglobulins in the glomerular capillaries, without morphological signs of cellular inflammation (12). Also in this model the glomerular pathology is accompanied with proteinuria.
Another model of antibody mediated vasculitis is that of the Nephrotoxic Nephritis (NTN), induced by injection of heterologous antibody directed against structures of the glomerular basement membrane (13). The morphology of the early glomerular lesion is that of an acute inflammation with accumulation of PMNs in the glomerular capillaries, resembling the vasculitis of the Arthus reaction (14,15). In the early, heterologous phase of the reaction binding of the antibodies to the glomerular basement membrane can be demonstrated ultrastructurally (13). In the second, autologous phase irregular deposits of electron dense material appear in the subendothelial region at the luminal side of the basement membrane. Both the heterologous and the autologous phase of the NTN are clinically accompanied with proteinuria. In the following discussions we will only refer to the early phase of the model, in which the heterologous antibody binds to the antigenic structures located in the basement membrane. With regard to its initiation by binding of antibody to structures that are fixed in the vessel wall the NTN shows a similarity to our model of AAR, in which an acute necrotizing vasculitis is induced by injection of specific antibody against antigenic determinants located on the vascular endothelium.

The striking resemblance of clinical forms of immune vasculitis to experimental acute serum sickness and the finding that in serum sickness, as well as in vascular lesions of other forms of immune vasculitis, immune complexes could be demonstrated both in the circulation and as depositions in the vessel walls, have led to the general assumption that most, if not all, types of immune vasculitis were caused by the deposition of circulating immune complexes in the vessel walls (5,7). In view of this concept, the hyperacute rejection of skin grafts by immunosuppressed mice, induced by intravenous injection of antibody that specifically binds to the endothelial cells of the graft, represents a model of immune vasculitis that can hardly be considered analogous to clinical immune complex disease. However, the concept that most types of immune vasculitis are caused by local "trapping" of preformed immune complexes has been seriously challenged recently, and there is accumulating evidence that local binding of immunoglobulins to fixed antigens plays a far more important role than has been previously assumed. Several

findings have made it necessary to modify the original concept of immune complex disease. In studies on the factors that determine the pathogenicity of circulating immune complexes it proved to be very difficult to induce experimental immune vasculitis by intravenous injection of preformed immune complexes (16). On the other hand, immune complexes could be demonstrated in the circulation of many patients without apparent immunovascular disease (16,17). Recent findings in models of immune vasculitis that formerly were thought to be prototypes of immune complex disease have also made this assumption questionable. For example, recent data suggest that both in the Passive Heymann Nephritis and in the chronic serum sickness the subepithelial deposits of immunoglobulins are not caused by local "trapping" of immune complexes, but are the result of binding of antibody to locally present intrinsic or planted antigens (18-20). The morphologic features of the glomerulopathy of the PHN and chronic serum sickness show a striking resemblance to those of clinical membranous nephropathy. Since also the occurrence of clinical membranous nephropathy could not be related to the presence of immune complexes in the circulation it is assumed that, analogous to the pathogenesis of PHN, the immune deposits in membranous nephropathy are the result of an in situ binding of antibody to fixed antigen (21). An indication that this may indeed be the case is the finding that DNA can bind in vitro to glomerular basement membrane and other collagenous structures (22), which could explain the occurrence of membranous neprhopathy in SLE, induced by binding of anti-DNA-antibodies.

Parallel to the changing views of the pathogenic potential of immune complexes the methods used for their identification in the serum have been criticized (23). Recent studies of our group on the presence of immune complexes in the serum of patients with rheumatoid arthritis indicate that some methods for detection of immune complexes in the serum, such as the C1q binding assay, induce complex formation of antigen and antibody in vitro, while in vivo the constituents are present in the uncomplexed state in the circulation (24). This also suggests that in vivo local factors may play an important role in determining whether or not complex formation will take place, and this could explain the predisposition of particular sites for immunologically induced inflammatory reactions.

In our opinion the above mentioned data, that suggests the occurrence of localized antigen-antibody reactions in experimental models and in clinical syndromes that previously were considered to be typical immune complex diseases, significantly increases the relevance of the model of AAR for the understanding of the pathogenesis of clinical vasculitis. It may be an important factor that in the AAR the antigen is located on the cell membrane of the vascular endothelium,

which means that in these circumstances the endothelial cells are not damaged as "innocent bystanders" of the inflammatory process, as is assumed in most of the above described models (25,26) but as the target cells of the direct attack of antibody. On the other hand, it is an obvious advantage of the model of AAR of skin grafts in mice that it enables us to define and manipulate the antigenic determinants, and thus to study the influence of antigenic structures on the development of vascular damage. The results of our experiments with AAR on skin allografts and xenografts in the mouse have shown that not only the occurrence of antibody-induced vasculitis, but also the morphology along which it develops and the degree in which effector systems such as complement are involved, are closely related to antigenic factors such as the number of antigenic targets and their accessibility for antibody in the circulation. Furthermore, our study on the expression of Ia antigens in mouse allografts has shown that the presence of these antigenic structures is related to the immunologic status of the host, and can vary with time within the same animal. Apart from the significance of these findings for our understanding of the physiologic role of Ia antigens on endothelial cells, further studies in this model may help to solve the question whether antibodies against HLA-DR (the human counterpart of the murine Ia antigens) have destructive capacity in clinical transplantation.

For the study of effector mechanisms the antibody induced vasculitis of AAR is clearly suitable, and it seems likely that insight in the pathogenic mechanisms of antibody-induced rejection can lead to a better understanding not only of clinical graft rejection, but also of other forms of clinical immunovascular disease, that show similarities in morphologic features. As mentioned before, classic candidates for effector roles in various models of immune vasculitis have been complement, PMNs, platelets, and the coagulation system. We will briefly discuss parallels between our findings in the AAR and those in the above mentioned other models of vasculitis.

The role of complement. There are several ways in which the complement system can act as effector mechanism in antibody-mediated cell damage. The first way is that of direct complement induced cell lysis, after activation of the complement cascade by the binding of antibody to antigenic structures on the target cell, and subsequent membrane attack by terminal complement components. The second way in which complement can mediate cell damage after activation by an antigen-antibody reaction is by attracting PMNs, which on their turn cause tissue damage by release of lysosomal enzymes during the process of phagocytosis of the immune complexes. The chemotactic attraction of PMNs is mainly effected by the factor C5a, and, to a lesser degree, the C567 complex, formed

during activation (27-29). This indirect effector role of complement in cooperation with PMNs has traditionally been assumed to be the most important one in the induction of immune vasculitis. A third way in which complement can possibly play a role in effecting cell damage is by induction of antibody mediated cell lysis by platelets. This mechanism has been shown to be effective in vitro, in an experimental setting in which antibody coated erythrocytes were incubated with platelets in the presence of early complement factors (30).

The role of complement in different models of immune vasculitis is not clear. The Arthus reaction and the early phase of the NTN are generally considered to be complement dependent and the main function of complement in these types of immune vasculitis is thought to be that of a chemoattractant for PMNs (15). However, in both these models it has been reported that very high doses of antibody can cause vessel wal damage in the absence of both complement and PMNs (3,14). The arteritis of serum sickness has also been reported to be complement dependent (6) but neither depletion of complement nor that of neutrophils prevents the development of glomerular lesions (31,32). Another role for complement, not as chemoattractant but independently from PMN activity, has been suggested in PHN, since in this model C3 depletion inhibits the proteinuria, whereas PMN depletion does not (33,34). This is consistent with the absence of PMN accumulation in the glomerular capillaries in this lesion. Therefore, complement must have another function here than that of producer of chemotactic factors. There is evidence that activation of the complete lytic pathway is required for the occurrence of the proteinuric effect (33,34). It has been reported that the terminal C5-C9 membrane attack complex of complement can also damage non-cellular membranous structures (35). Still, how complement effects its mediation in the PMN-independent induction of damage to the glomerular capillaries in PHN remains so far unclear.

Opinions on the role of complement and its way of action in the AAR have been controversial. Our findings, as presented in this thesis, have clarified two points in the discussion. The first is that the antibody induced vasculitis of the AAR cannot be simply described as being complement dependent or complement independent. The second is that the action of complement in the AAR is not directly related to its chemotactic attraction of PMNs. Our results have shown that the damaging potential of antibody, as determined by its dose on the one hand and the number of available targets on the other, can determine whether or not, or to what degree the resulting vasculitis is complement dependent. In murine allografts for instance, where the histocompatibility disparities are limited, and consequently antigenic targets on

the endothelium are relatively sparse, the AAR is clearly dependent on the availability of an effective complement system. In this combination of donor and recipient the weakly acting endogenous complement system of the mouse is not sufficient to induce AAR after administration of specific antibody, not even when the injected dose is very high (36). In these mouse skin allografts AAR can only be elicited when together with the antibody a more efficient heterologous complement is injected, such as guinea pig complement, human complement, or, the most efficient one, rabbit complement (37,38). In the xenograft model, in which more abundant antigenic targets are available for binding with the antibody, the murine complement is efficient enough for induction of AAR (39), and in this combination AAR can even be induced in the absence of complement, provided that the dose of antibody is substantially raised (40,41). The finding that neither the Shwartzman-like nor the Arthus-like type of AAR was prevented or inhibited by PMN depletion made it clear that in the AAR activated complement does not act via attraction of PMNs, and consequently we now favor the opinion that in the presence of complement the initial damage to the endothelial cells of the graft is that of a direct antibody-mediated cell lysis, caused by activation of the complement cascade. In this respect the AAR is different from the other models of immune vasculitis in which the antigen is not located on the endothelial cell membrane, but either deposited or fixed onto vessel wall structures, or present as a soluble factor in blood or interstitial fluid.
In these cases complement-dependent tissue damage must be effected either by secondary inflammatory mechanisms, or, as mentioned above, by complement-induced damage to non-cellular membranous structures.

The way in which in the AAR vessel wall damage is induced in the absence of complement remains so far unclear. Our initial hypothesis that in complement depleted or complement deficient recipients PMNs, under these circumstances attracted by other chemotactic factors than complement components, were responsible for the initial vessel wall damage, was no longer tenable after our subsequent finding that PMN depletion did not prevent the AAR in the absence of complement. It could be argued that very low, unmeasurable traces of complement are still present locally in Cobra Venom Factor treated animals, and that these minimal amounts of complement could be sufficient to induce endothelial cell lysis in the presence of the very large doses of antibody, that are necessary in these circumstances to induce AAR. However, in congenitally C5 deficient mice the complement cascade cannot be fully activated, and in these circumstances other effector systems that are either complement-independent, or that only require minimal amounts of early complement components, must be operative.

The role of granulocytes. The characteristic and dominating presence of polymorphonuclear granulocytes in many types of immunologically induced inflammatory processes suggests that these cells have an important role in the mediation of the resulting tissue damage. This is generally thought to be effected by the release of lysosomal enzymes during the process of phagocytosis of immune complexes. The migration of granulocytes to the reaction site is caused by chemotactic factors, either serum derived, cell derived, or produced by infecting micro-organisms such as bacteria. The most effective chemotactive activity is thought to be derived from the complement system and especially C5a has been shown to be a major humoral chemotactic factor (27). A PMN-dependency of immunologically induced inflammatory reactions is therefore often related to complement-dependency, as has been discussed above.

As mentioned before we formerly assumed that PMNs were involved in the AAR, especially in the Arthus-type, in a similar way as in the Arthus reaction, i.e. that in this type of AAR granulocytes were essential mediators for the tissue damage. In complement depleted animals we supposed that granulocytes could act by adherence to the antibody coated endothelial cells by means of their Fc receptors. However, our results in PMN depleted recipients show that the AAR is not PMN dependent, neither in complement normal, nor in complement deficient mice. In this respect the model of AAR is essentially different from both the Arthus reaction and the NTN, while it shares its PMN-independency with the PHN.

The role of platelets. In general, platelet aggregation and intravascular coagulation are not characteristic features of the early phases of immunovascular diseases, including those of serum sickness, NTN, and PHN, and there is no indication that platelets play an important role in these processes. In the Arthus reaction platelet aggregation and intravascular coagulation occur in the later stages of the process, and, although the obstruction of the circulation may increase the tissue damage, it has been shown that depletion of platelets or treatment with heparin does not prevent the reaction (3). In the AAR, platelet aggregation and intravascular coagulation can be the first and most impressive sign of vascular destruction, thus causing a resemblance of the vasculitis to that of the generalized Shwartzman reaction. Assuming that in this type of AAR the damaging potential of antibody and complement is sufficient to cause immediate endothelial cell lysis, the early platelet aggregation, caused by denuding of the vascular surface, could be an important secondary pathway that augments the resulting tissue necrosis. If, however, contrary to our hypothesis, the inital event in the vasculitis of AAR is not a direct complement induced endothelial cell destruction, platelets could possibly play a role as initiators of the inflammatory process by means of platelet induced lysis of antibody coated cells, dependent on the

presence of early complement factors (30). We mentioned this mechanism as one of the ways in which complement can mediate cell damage. There is some evidence that this lytic effect of platelets may also be effective in vivo (30).

Preliminary results of experiments in which we induced AAR in platelet depleted and heparin treated recipients have shown that these treatments do not prevent the occurrence of AAR in complement normal mice, neither the Arthus nor the Shwartzman type. Moreover, platelet depletion did not change a Shwartzman-like type of AAR into an Arthus-like one, as we had expected. In these mice we observed, after injection of high doses of antibody, rapid rejection of all grafts similarly to that in control groups with normal platelet counts. Histologic sections of the grafts showed, in the first two hours after injection of antibody, vessel wall damage and extensive bleeding, with absence of platelet aggregation and PMN accumulation. Also in mice that received low doses of antibody and in which the control groups showed an AAR of the Arthus type, platelet depletion did not alter the eventual fate of the grafts, although the initial patchy bleeding was more prominent. Thus far we have not tested a possible effector role for platelets in complement depleted or complement deficient animals. Since only early complement factors are necessary for the supposed lytic effect of platelets on antibody bearing cells, testing of platelet depletion in C5 deficient mice will not be sufficient. Cobra Venom Factor treatment, on the other hand, implies that daily i.p. injections have to be given to platelet depleted animals, which will cause severe bleeding, leading to great losses and, therefore, possibly also to non-specific interference with graft rejection. Other, non-invasive methods of complement depletion must be developed before this hypothesis can be adequately tested in vivo. For the present we will have to confine ourselves to test the role of platelets in the absence of complement in an in vitro system.

Other effector mechanisms. Apart from the classic candidates, viz. complement, granulocytes, and platelets, for effector roles in immune vasculitis also other mechanisms for the initial induction of vessel wall damage have to be considered. One of these could be antibody-dependent cell-mediated cytotoxicity (ADCC), effected by lymphoid cells or monocytes and macrophages, that can adhere to antibody-bearing cells by means of their Fc receptors. From the histologic sections we could gather no indications that mononuclear cells play a significant role in the initiation of the vasculitis of the AAR, neither in complement normal nor in complement deficient mice. Also other experimental data to support a destructive role for macrophages in the initial phase of antibody-induced immune vasculitis are lacking. However, this does not exclude a possible effector role for these cells.

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SUMMARY

For the study of the histologic events in the vasculitis of the acute antibody-mediated graft rejection (AAR), and the role of mediators in this process, a model was chosen in which rat skin was grafted onto the flanks of immunosuppressed mice. At day 7 or 8 after transplantation, when the grafts were well healed and in excellent condition, acute antibody-mediated rejection was induced by intravenous injection of mouse anti-rat lymphocyte serum. It was demonstrated that the histologic pattern of graft destruction was dependent on the amount of antiserum administered. After injection of low doses of antibody a rejection was seen that was histologically characterized by early accumulation of granulocytes in the graft vessels, at later stages followed by vessel wall damage, intravascular platelet aggregation and necrosis. This pattern of vasculitis resembled that of an Arthus reaction. After injection of high doses of antibody another type of acute vasculitis occurred, characterized by early intravascular platelet aggregation and coagulation, thus closely resembling the vasculitis of the generalized Shwartzman reaction. In mice that received intermediate doses of antibody the grafts showed changes that were transitional between these two types of vessel wall destruction. In other experimental models and in clinical transplantation similar histologic patterns of acute antibody-mediated rejection have been described, and, based on different histologic findings, controversial conclusions have been drawn on the role of mediators in the induction of antibody-mediated graft destruction.
Our results show that the pattern along which the vasculitis of the AAR develops can be a variable one, and can fully depend on the amount of antibody that reaches the graft, i.e. on the severity of the triggering reaction.

In a second set of experiments the role of complement in the vasculitis of the AAR was studied by testing the effect of complement depletion on the occurrence of the rejection, and on the histologic pattern along which the vasculitis develops. It was found that depletion of C3 by treatment with Cobra Venom Factor inhibited the AAR after injection of low doses of antibody. However, C3 depletion did not prevent the occurrence of AAR after intravenous injection of high doses of antibody, but in the absence of complement the reaction in the vessels changed from a Shwartzman-like type of vasculitis into

an Arthus-like one. Conversely, supplementary administration of rabbit complement caused a violent Shwartman-like graft destruction after injection of low doses of antibody, that in complement normal mice gave a more slowly developing Arthus-like reaction. We conclude that not only the amount of antibody but also the availability of complement can be the decisive factor that determines whether or not acute antibody-mediated rejection will occur and along which histological pattern the vasculitis will take place. When large doses of antibody are injected, however, complement is not an absolute requirement for the occurrence of antibody-mediated graft destruction.

The histologic resemblance of the vasculitis of the AAR occurring either after injection of low doses of antibody or in the absence of complement, to the experimental Arthus reaction suggested an essential role for polymorphonuclear granulocytes in the initiation of the vessel wall damage. On the other hand the morphology of the AAR which occurred after injection of high doses of antibody or in the presence of abundant amounts of highly effective complement, and which developed along a Shwartzman-like type of vasculitis, suggested that PMNs did not play an important role in the early phases of the process.
Therefore, the role of granulocytes in either type of vasculitis was tested by inducing AAR in recipients depleted of PMNs. Since conventional methods to induce granulocyte depletion, such as treatment with Nitrogen Mustard or anti-granulocyte serum, proved to be insufficient to effect a stable PMN depletion in mice for several days, and were accompanied by considerable toxic side effects, we induced PMN depletion by Total Body Irradiation (TBI).
This irradiation was given in a single dose of 6.0 Gy (600 rad), at a dose rate of 0.20 Gy min^{-1}. To prevent infection, the mice were kept in a laminar flow unit from two days before until 3 weeks after the irradiation, under an aseptic regimen and protective antibiotic treatment. The TBI of 6.0 Gy reduced the number of PMNs in the peripheral circulation of the mice to levels below 150/µl from 3 to 10 days after the irradiation, without causing significant changes in complement levels. The number of lymphocytes dropped simultaneously with that of granulocytes, but platelet counts remained above 60% of normal values in the first 7 days after the irradiation. Furthermore, the TBI, under the aseptic regimen, did not visibly impair the general condition of the mice.

To investigate the effect of PMN depletion on the AAR of rat skin xenografts, mice received a TBI of 6.0 Gy on day 4 after transplantation, and mouse anti-rat globulin was injected intravenously on day 8 after transplantation.
Our results show that neutrophil depletion did not prevent the

AAR, neither that of the Shwartzman type, nor that of the Arthus type. Moreover, we found that AAR could be elicited in PMN depleted recipients that were complement depleted by treatment with Cobra Venom Factor, or that were congenitally C5 deficient. We concluded that granulocytes are not essential mediators in the antibody-induced rejection of skin grafts. This conclusion was further supported by the finding that also in mouse skin allografts, in which the AAR always develops along an Arthus-like pattern of vasculitis, AAR could be induced after PMN depletion of the recipients. For this study immuno-suppressed B10.D2 recipients of B10.A skin grafts received TBI of 6.0 Gy on day 4 after transplantation, after which, on day 8 after transplantation, AAR was induced by intravenous injection of specific monoclonal antibody together with rabbit complement.

Our finding that PMNs, although they act as accelerators and amplifiers of the acute antibody mediated rejection process, are not essential mediators in the initial vessel wall damage, led us to reconsider the function of these cells in those types of AAR that morphologically develop along an Arthus-like pathway, and especially to revise our assumption that in complement depleted or complement deficient mice AAR is effected by the abundantly present granulocytes. In general our results are consistent with the hypothesis that the initial damage of the antibody to the endothelial cells is due to a direct membrane attack of complement, caused by its activation after binding of the antibody to the antigenic targets located on the endothelial cell membrane. However, this hypothesis does not explain how antibody-induced vessel wall damage is effected in complement depleted or complement deficient animals.

The general distribution of Class I antigens, including their presence on endothelial cells, explains why the vessels of the graft are the primary target for both the cellular and the antibody-mediated rejection process. In man also Class II antigens have been detected on the vascular endothelium. In the mouse, however, Ia antigens were thought to be restricted to epidermal Langerhans cells, and consequently these cells were supposed to be the target cells for rejection of mouse skin grafts across Ia disparities. We investigated the localisation of Ia antigens in mouse skin allografts by in vivo injection of labelled monoclonal anti-Ia antibodies in Ia-incompatible donor-recipient combinations. The in vivo binding of anti-Ia antibody in the grafts was measured by counting the radioactivity in grafts that were excised at different times after injection of the antibody, and the localisation of the antibody in the grafts was visualized by immunofluorescence and autoradiography. In these studies we could demonstrate Ia antigens on the endothelial cells of mouse skin allografts. However, their expression on these

cells is variable and depends on the immune status of the recipient. These findings suggest that the vascular endothelium can act as target in Ia-incompatible skin allograft rejection.

Study of the mechanisms involved in the vasculitis of the antibody-mediated rejection will not only lead to a better understanding of acute and chronic forms of antibody-mediated rejection, but will also give more insight in the pathogenesis of other forms of antibody-induced vasculitis. It has been generally assumed that most forms of clinical and experimental immune vasculitis were caused by deposition of immune complexes in the vessel walls.
Recent data, however, suggest that binding of antibody to fixed antigens plays an important role in the initiation of several forms of immune vasculitis that were formerly thought to be prototypes of diseases caused by circulating immune complexes, and analogies to related clinical immune complex diseases have been drawn. This data increases the relevance of the model of AAR for the study of antibody-induced vasculitis.

HYPERACUTE ANTILICHAAM-AFHANKELIJKE TRANSPLANTAAT-AFSTOTING ALS MODEL VOOR IMMUUNVASCULITIS

De morfologie van de vasculitis van de hyperacute antilichaam-afhankelijke transplantaat-rejectie (AAR), en de rol van mediatoren in dit proces, werd bestudeerd in een model waarin PVG/c rattehuid werd getransplanteerd op de flank van C57Bl/10 muizen, die, ter voorkoming van cellulaire afstoting, behandeld werden met anti-lymfocyten serum. Op dag 7 of 8 na transplantatie werd hyperacute afstoting opgewekt door intraveneuze toediening van muis-anti-rat serum.
Bij histologisch onderzoek bleek dat het patroon waarin de vasculitis van de acute afstoting zich ontwikkelde afhankelijk was van de dosis toegediend antiserum: wanneer lage doses werden ingespoten zagen we een afstotingsproces dat morfologisch gekenmerkt werd door een vroeg optredende accumulatie van granulocyten in de transplantaatvaten, in latere stadia gevolgd door het optreden van vaatwandbeschadiging, intravasale stolling en necrose. Dit type vaatreactie lijkt sterk op dat van de experimentele Arthus-reactie. Na het toedienen van hoge doses antiserum trad een ander type vasculitis op, dat vooral gekenmerkt werd door een vrijwel onmiddellijk optredende intravasculaire plaatjes-aggregatie en stolling, een beeld dat ook gezien wordt bij de experimentele gegeneraliseerde Shwartzman-reactie. Na het toedienen van tussenliggende doses antiserum zagen we mengvormen optreden van beide genoemde typen van vasculitis.
De bovengenoemde patronen van vaatreactie zijn ook beschreven bij hyperacute antilichaam-afhankelijke transplantaat-rejectie in andere diermodellen, en in klinische afstotings-reacties. De opvallende morfologische verschillen tussen vasculitisvormen van het Arthus-type en van het Shwartzman-type, en de verschillende rol die granulocyten en plaatjes lijken te spelen m.n. in de vroege fase van de reactie, hebben geleid tot tegenstrijdige theorieën over de rol van mediatoren in de pathogenese van de antilichaam-afhankelijk afstoting. Onze resultaten tonen aan dat de Arthus-achtige en de Shwartzman-achtige vormen van vasculitis niet beschouwd moeten worden als twee pathogenetisch verschillende reacties, maar als onderdelen van één morfologisch spectrum van vaatwandbeschadiging.

Hierbij kan de hoeveelheid toegediend antiserum de factor zijn die bepaalt welk type reactie zal optreden.

De rol van complement in de AAR werd getest door hyperacute afstoting van rattehuidtransplantaten op te wekken bij muizen waarin de lytische aktiviteit van complement werd uitgeschakeld d.m.v. behandeling met Cobra Venom Factor. Het bleek dat bij ontbreken van complement-aktiviteit hyperacute afstoting slechts kon worden opgewekt wanneer hoge doses antiserum werden toegediend. Bovendien bleek dat het morfologisch patroon van de vasculitis die optrad na inspuiten van deze hoge doses, door het ontbreken van complement, veranderde van een fulminant verlopend Shwartzman-type in een minder snel verlopend Arthus-type. Omgekeerd konden we d.m.v. extra toediening van efficient heteroloog konijne-complement een afstoting volgens een Arthus-patroon, zoals optreedt na toediening van lage doses antistoffen, doen veranderen in een reactie volgens een heftig verlopend Shwartzman-patroon. Uit deze experimenten bleek dat zowel het optreden van de AAR, als het patroon waarlangs de vaatontsteking verloopt, niet alleen wordt bepaald door de dosis toegediend antiserum, maar ook door de mate waarin complement beschikbaar is. Hiermee worden ook de resultaten van eerdere experimenten bevestigd, waaruit bleek dat het complement-systeem weliswaar funktioneert als een belangrijk amplificatie-mechanisme in het ontstaan van vaatwand-destruktie, maar dat het niet onmisbaar is voor het optreden van AAR.

Het feit dat AAR in sommige gevallen verloopt volgens een vasculitispatroon dat sterk lijkt op dat van de Arthus-reaktie, en in andere gevallen volgens een beeld van acute vaatwanddestruktie, zoals gezien wordt bij de gegeneraliseerde Shwartzman-reactie, heeft geleid tot verschillende, tegenstrijdige hypothesen over de pathogenese van de vaatwand-beschadiging, waarin enerzijds aan granulocyten, anderzijds aan plaatjes en het stollingssysteem een belangrijke rol werd toegeschreven bij de induktie van de primaire vaatwand-beschadiging. Doordat we in ons model van hyperacute afstoting van xeno-transplantaten bij de muis zowel een Arthus- als een Shwartzman-type van vasculitis konden opwekken, was het mogelijk om de rol van mediatoren te testen in beide vormen van vasculitis afzonderlijk. Voor het bestuderen van de rol van granulocyten testten we het effect van granulocyten-depletie op het ontstaan van AAR. Aanvankelijk werd getracht granulocyten-depletie bij de muizen op te wekken met vaak gebruikte methoden zoals behandeling met stikstofmosterd of met anti-granulocyten serum. Deze behandelingen leidden echter niet tot een reproduceerbare granulocyten-depletie, waarbij het aantal segmentkernigen in de circulatie gedurende enkele dagen beneden het niveau van 150/µl gehouden kon worden. We trachtten daarom dit effect te bereiken door totale lichaamsbestraling. Deze werd gegeven

als een eenmalige dosis van 6,0 Gy (600 rad), toegediend over een tijdsduur van 30 min. Om intestinale complicaties te voorkomen werden de muizen vanaf 24 uur vóór de bestraling nuchter gehouden, en ter voorkoming van infectie werden de dieren vanaf twee dagen vóór bestraling gehuisvest in een laminar flow unit, onder aseptische omstandigheden, waarbij ze gesteriliseerd voer ontvingen en profylactisch antibiotica kregen toegediend in het drinkwater. Onder deze omstandigheden bleken de muizen de totale lichaamsbestraling uitstekend te verdragen. De dosis van 6,0 Gy induceerde een granulocyten depletie die stabiel en reproduceerbaar bleek, en waarbij het aantal segmentkernigen in de perifere circulatie van dag 3 tot 10 na bestraling beneden het niveau van 100/µl lag.

De invloed van granulocyten depletie op het optreden van AAR werd vervolgens bestudeerd bij muizen die op dag 4 na transplantatie van de rattehuid een totale lichaamsbestraling ondergaan hadden. Op dag 8 na transplantatie (dag 4 na bestraling) werd bij deze muizen intraveneus anti-rat serum toegediend in verschillende doseringen. De resultaten van deze experimenten tonen aan dat granulocyten-depletie het optreden van AAR niet kon voorkomen, noch die van het Arthus-type, noch die van het Shwartzman-type. Bovendien bleek dat ook in muizen die behandeld waren met Cobra Venom Factor, of die congenitaal C5-deficient waren, AAR kon worden opgewekt in afwezigheid van granulocyten.
We concludeerden hieruit dat granulocyten geen essentiele mediatoren zijn in het proces van vaatwandbeschadiging bij de AAR. Deze conclusie werd ondersteund door het resultaat van experimenten waarin bij granulocyten-deplete muizen AAR van allo-transplantaten werd opgewekt. In dit allogene model werd bij de ontvangers, nadat zij op dag 4 na transplantatie een totale lichaamsbestraling van 6,0 Gy hadden ondergaan, op dag 8 na transplantatie specifiek tegen het transplantaat gericht monoclonaal antiserum ingespoten te zamen met konijne-complement. Ook in zulk een donor-ontvanger combinatie, waarin de afstoting na toediening van specifiek allo-antiserum en complement altijd verloopt volgens een Arthus-patroon, bleek AAR op te treden ondanks de afwezigheid van granulocyten.

Op basis van bovengenoemde experimenten hebben we onze oorspronkelijke veronderstelling dat granulocyten een belangrijke rol spelen in het ontstaan van de primaire vaatwandbeschadiging in de AAR, met name in die van het Arthus-type, moeten herzien. De resultaten van onze experimenten ondersteunen de hypothese, dat de primaire vaatwand-beschadiging in de AAR ontstaat door een direkte complement-afhankelijke lysis van de endotheel cellen, veroorzaakt door aktivatie van complement t.g.v. de binding van antilichaam aan antigenen, die gelokaliseerd zijn op de

celmembraan. Aan de andere kant verklaart deze hypothese niet het optreden van AAR bij complement-deficiente of met Cobra Venom Factor behandelde ontvangers.

Het feit dat het endotheel van de transplantaatvaten fungeert als doelwit voor zowel de cellulaire als de antilichaam-afhankelijke afstotingsreactie kan verklaard worden door de algemene aanwezigheid van Klasse I antigenen op alle cellen. Klasse II antigenen zijn echter niet op alle cellen aanwezig, en waren tot dusverre niet aangetoond op endotheel van de muis. Omdat in muizen toch afstoting van allo-transplantaten optreedt over Ia-verschillen werd aangenomen, dat deze afstoting werd geinduceerd door Ia-antigeen dragende Langerhans cellen aanwezig in het transplantaat. In een experimenteel model waarin we trachtten de Ia-antigenen in het transplantaat aan te tonen d.m.v. het in vivo inspuiten van radioactief gelabelde monoclonale anti-Ia antilichamen bij dragers van Ia-incompatibele transplantaten, vonden we, dat onder bepaalde omstandigheden Ia-antigeen ook kon worden aangetoond op het transplantaat-endotheel. De expressie van deze antigenen bleek nauw samen te hangen met de immuunstatus van de ontvanger. Bij muizen die behandeld waren met antilymfocyten serum, of Cyclosporine A, of waarbij een congenitale-immuundeficientie bestond gecombineerd met het congenitaal ontbreken van de thymus, was geen expressie van Ia-antigenen op het endotheel aantoonbaar. Bij muizen echter, waarbij de immuun-reactiviteit intakt was, kon met immunofluorescentie en autoradiografie Ia-antigeen worden aangetoond op het endotheel van de transplantaten. Kwantitatieve meting van de binding van gelabelde anti-Ia-antistof werd verricht door het tellen van de radioaktiviteit in de transplantaten op verschillende tijdstippen na het inspuiten van het antiserum. De resultaten van deze experimenten suggereren dat het vaatwand-endotheel, als drager van Ia-antigeen, kan fungeren als rechtstreeks doelwit in de afstoting over Ia-verschillen.

Het bestuderen van de pathogenetische mechanismen in de vasculitis van de acute antilichaam-afhankelijke afstoting is niet alleen van belang om een beter inzicht te krijgen in de processen die leiden tot acute of chronische antilichaam-afhankelijke transplantaatbeschadiging, maar kan ook het inzicht vergroten in andere vormen van vasculitis die veroorzaakt worden door antilichamen. Hoewel tot voor kort algemeen werd aangenomen, dat de meeste vormen van klinische en experimentele immunologisch geinduceerde vaatwand-ontstekingen veroorzaakt werden door het neerslaan of gevangen worden van immuuncomplexen in de vaatwand, is op grond van recente studies deze veronderstelling in twijfel getrokken. Het is gebleken dat in sommige vormen van experimentele immuun-vasculitis, die vroeger beschouwd werden

als typische voorbeelden van immuuncomplex-ziekten, de primaire beschadiging van de vaatwand niet wordt veroorzaakt door neerslagen van circulerende immuuncomplexen, maar door binding van antilichaam aan antigeen dat gelokaliseerd is in de vaatwand, hetzij doordat het daar intrinsiek aanwezig is, hetzij doordat het tevoren daar gedeponeerd en gefixeerd is. Deze bevindingen hebben een nieuw licht geworpen op de pathogenese van immuunvasculitis en analoog aan de bevindingen in experimentele modellen wordt nu aangenomen, dat ook in klinische vormen van immuunvasculitis de binding van antilichaam aan gefixeerd antigeen een veel grotere rol speelt, dan voorheen verondersteld werd. Deze gegevens vergroten het belang van de bestudering van de pathogenese van de vaatbeschadiging in de acute antilichaam-afhankelijke transplantaat afstoting als middel tot het verkrijgen van een beter inzicht in de pathogenese van andere vormen van antilichaam-afhankelijke vasculitis.

Velen hebben meegewerkt bij het verrichten van het onderzoek en bij het tot stand komen van dit proefschrift. In de eerste plaats wil ik hier noemen Ine Cornelissen die een belangrijke rol speelde in het dierexperimenteel werk. Zij blonk hierbij met name uit door snel en fraai transplanteren en door de fabelachtige trefzekerheid waarmee ze muizen intraveneus kan spuiten. Cathy Maass ben ik erkentelijk voor het maken van de vele histologische coupes. Door de constant hoge kwaliteit zou men bijna vergeten hoe moeilijk het snijden van muizehuidjes is. Behalve op deze twee naaste medewerkers heb ik, wanneer vele handen nodig waren, altijd met succes een beroep kunnen doen op Tiny Tangelder. Jacqueline Hagemann verrichtte de complement bepalingen en was bovendien steeds bereid een helpende hand te bieden. Bij de dierexperimenten werd nooit vergeefs een beroep gedaan op de vakkundige en vriendschappelijke hulp van de medewerkers van het Centraal Dierenlaboratorium. Met name Jan Koedam en Lia van de Vorle-Houben ben ik erkentelijk voor hun hulp en advies bij het huisvesten van de bestraalde muizen. Ook de medewerkers van de experimentele boerderij in Overasselt wil ik graag mijn dank betuigen. De medewerkers van de Afdeling Radiotherapie wil ik bedanken voor de soepelheid waarmee tijd en ruimte werd vrijgemaakt voor onze muizen, en voor de zorgvuldigheid en efficientie waarmee de bestraling werd uitgevoerd. Ook ben ik blij hier mijn dank te kunnen betuigen aan de medewerkers van de Afdeling Medische Fotografie, die op vakkundige wijze de foto's verzorgd hebben, en die altijd bereid zijn tot het geven van advies. Op gelijke wijze ben ik dank verschuldigd aan de medewerkers van de Afdeling Medische Illustratie. De medewerkers van het laboratorium Kindergeneeskunde stelden hun apparatuur ter beschikking voor het tellen van bloedcellen en -plaatjes. De heer E. de Graaff en medewerkers van de Centrale Bibliotheek, Gerry Hermkens en Anita de Vries hebben geholpen bij het verzamelen van de literatuur. Tenslotte wil ik Erna Franssen-Kokke, Anita de Vries en Janny van Rennes bedanken voor het typen van de artikelen, en Janny van Rennes nog eens speciaal voor het persklaar maken van het manuscript.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 10 juli 1939 te Brunssum. Zij doorliep het Gymnasium β op het Serviam Lyceum te Sittard, waar zij in 1957 het einddiploma behaalde. Daarna studeerde zij Geneeskunde aan de Katholieke Universiteit van Nijmegen en legde in 1963 het doctoraal examen en in 1966 het artsexamen af. Vanaf januari 1973 was zij in opleiding tot Patholoog Anatoom op de Afdeling Pathologische Anatomie van het Sint Radboud Ziekenhuis te Nijmegen (hoofd van de opleiding Prof.Dr. P.H.M. Schillings), en zij werd ingeschreven in het specialisten register op 1 januari 1977 Vanaf die datum is zij werkzaam als wetenschappelijk medewerker op dezelfde afdeling, waar zij naast de algemene taak in opleiding en onderwijs in het bijzonder belast is met de diagnostiek van de maligne lymfomen. Het wetenschappelijk onderzoek, voor zover dit niet direkt klinisch gericht is, concentreert zich op de transplantatie-immunologie en wordt verricht in een samenwerkingsverband van de Afdeling Pathologische Anatomie met de Afdeling Nierziekten van de Kliniek voor Inwendige Ziekten.

STELLINGEN

I

Uit de opvallende intravasculaire aanwezigheid van granulocyten in de eerste fase van acute antilichaam-afhankelijke rejectie is ten onrechte geconcludeerd dat deze cellen een essentiële rol spelen in het initiëren van de vaatwandbeschadiging.

II

Totale lichaamsbestraling is tot dusverre de meest geschikte methode om granulocytendepletie op te wekken bij de muis.

III

Bij klinische immuunvasculitis speelt de reactie van antilichamen met in de vaatwand gefixeerde antigenen naar alle waarschijnlijkheid een veel belangrijker rol dan tot dusverre verondersteld werd.

IV

Hoewel het prognostisch belang van de morfologische typering van de ziekte van Hodgkin door de aanzienlijk gestegen overlevings-kansen van alle typen duidelijk is afgenomen, moet het gebruik van deze typering door pathologen, met in acht nemen van strikte criteria, gehandhaafd blijven om de huidige goede diagnostische accuratesse bij het stellen van de primaire diagnose niet verloren te doen gaan.

V

Het histologisch beeld van Hairy Cell leukemie in de milt kan, wanneer het karakteristiek en volledig is, als pathognomonisch voor deze ziekte beschouwd worden.

VI

Voor optimale hematologische beoordeling van in plastic ingebedde cristabiopten is het noodzakelijk dat de patholoog zijn bevindingen kan correleren met die in het uitstrijkpreparaat.

VII

Wanneer bij een patient met een testistumor de serumwaarden van β-choriogonadotropine en α-foetoproteine verhoogd zijn moet de tumor als een niet-seminomateuze testistumor behandeld worden, ook wanneer histologisch alleen een seminomateuze component kan worden aangetoond.

VIII

De monoclonale antistof WT1 is niet alleen een veelbelovend immunosuppressivum, maar is ook een belangrijke aanwinst als T-cel marker in de diagnostiek van de acute lymfatische leukemie en het lymfoblastair lymfoom.

IX

Hoewel phase-contrast optiek de vormveranderingen van erythrocyten in de urine bij een glomerulaire hematurie het fraaist zichtbaar maakt, voldoet microscopie met een normale belichting en in lage stand geplaatste condensor uitstekend voor de routine-diagnostiek.

X

De ontwikkeling van de wetenschap heeft voldoende aangetoond dat "maatschappelijke relevantie" als selectie-criterium bij de beoordeling van wetenschappelijk onderzoek onbruikbaar is.

XI

Wanneer men de som van de frekwenties van rectumcarcinoom en prostaatcarcinoom bij de man vergelijkt met de frekwentie van mammacarcinoom of cervixcarcinoom bij de vrouw is het verwonderlijk dat er in het afgelopen decennium geen voorstellen zijn gedaan om alle mannen boven 50 jaar op te roepen om zich naar een in hun buurt gereedstaande bus te begeven en zich daar, tegen geringe vergoeding, door speciaal daarvoor opgeleide paramedische krachten rectaal te laten toucheren.

XII

Het irreversibel mislukken van een Sauce Béarnaise kan grotendeels voorkomen worden door de zuurgraad van de waterige vloeistof, die de basis vormt van de emulsie, zo laag mogelijk te kiezen.

D.M.Small en M.Bernstein. Doctor in the Kitchen: Experiments on Sauce Béarnaise. N Engl J Med 1979, 300: 801-807.

Nijmegen, 16 december 1983 M.J.J.T. BOGMAN