ACUTE DESTRUCTION BY HUMORAL ANTIBODY OF RAT SKIN GRAFTED TO MICE

THE ROLE OF COMPLEMENT AND POLYMORPHONUCLEAR LEUKOCYTES*

BY HENRY J. WINN, CONRAD A. BALDAMUS, SOMARIE V. JOOSTE, AND PAUL S. RUSSELL

(From the Transplant Unit, General Surgical Services, and the Department of Surgery, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts 02114)

(Received for publication 29 December 1972)

We have described briefly the acute destruction of rat skin xenografts after injections of their hosts with antisera specifically reactive with graft antigens (1). The rat skin had been grafted to mice whose immune responses were suppressed by removal of the thymus and treatment with rabbit antimume lymphocyte serum. When these xenografts had healed completely into their beds and could be fully expected to survive for one or more additional weeks, the mice were injected with antirat serum that had been prepared in mice or in rabbits. Signs of graft damage were evident as early as 10 rain after the injection of serum, and in most animals so injected the grafts were completely destroyed within 24-48 h. Studies in which fixed sections of grafts were examined microscopically revealed the early occurrence of edema and infiltration of the grafts with polymorphonuclear leukocytes followed by extreme dilatation and congestion of small vessels, hemorrhage, and necrosis (2). Both gross and microscopic aspects of these reactions were similar to those described for Arthus reactions and other forms of immune vasculitis in rabbits and guinea pigs (3, 4). Reactions of the latter types have been the subject of intensive investigation in recent years, and a good deal has been learned of the pathways leading to tissue damage in these reactions (5). We have looked, therefore, for similar mechanisms in the destruction of skin grafts that is mediated by humoral antibodies, and the experiments to be described here were designed primarily to evaluate the roles of granulocytes and the plasma complement system, agents which are known to be involved in a variety of lesions that are initiated through immunological reactions.

Materials and Methods

Experimental System.—The basic test system has been described in detail (2). Adult mice were thymectomized 1–2 wk before receiving grafts of rat ear skin. Rabbit antimouse lympho-

* This study was supported by U.S. Public Health Service grants AI-06918, AI-06320, and AM-07055.
† Recipient of a Stipend from the Deutsche Forschungsgemeinschaft.
cyte serum (RAMLS) was injected intraperitoneally at a dose of 0.25 ml 2 days before grafting, on the day of grafting, and on days 2 and 4 after placement of the grafts. About 2 wk after transplantation when the grafts were well vascularized and in excellent condition, antisera or fractions of antisera specifically reactive with graft antigens were injected intravenously or intraperitoneally (i.p.). The grafts were observed for signs of inflammation (edema and hyperemia) and epithelial damage. Rejection was considered complete when no viable donor epithelium could be detected macroscopically.

Animals.—Male and female rats of the following inbred lines were purchased from Microbiological Associates, Inc., Bethesda, Md., and used as sources of ear skin for grafting: Brown Norway, Lewis, and Fischer. The recipients of the grafts were mice of the inbred lines B10-D2 new and B10-D2 old purchased from the Jackson Laboratory, Bar Harbor, Maine. These two lines are congenic with each other as well as with C57BL/10 Sn (6). The only known genetic difference between the old and new B10-D2 lines relates to the alleles carried by them at the Hc loci (6). Genes at this locus control the synthesis of the fifth component of complement (C5), and there are currently two alternative or allelic forms of the genes that are detectable. B10-D2 new is homozygous $h^c/h^c$ and has detectable levels of C5 and hemolytic complement. B10-D2 old is homozygous $h^c/0/h^c/0$. These animals have no detectable C5 as measured by functional or immunochemical tests, and therefore, lack hemolytic complement (7). Comparative studies with these congenic lines provide a means of evaluating the role of C5 and later-acting components of complement in vivo. Female mice were used exclusively in all experiments except those summarized in Table IV B; only males were used in the latter. A/J and B6AF1 hybrid female mice were also purchased from the Jackson Laboratory and used in the preparation of mouse antirat serum (MARS) or as graft recipients in the initial experiments that demonstrate the immunosuppressive properties of RAMLS and the destructive properties of antirat sera.

Antisera.—Rabbit antirat serum (RARS), RAMLS, and MARS were prepared as described previously (2). In each case animals were injected first with lymphoid cells in complete Freund’s adjuvant and subsequently with cells suspended in saline. The major portion of the MARS was prepared in B6AF1 hybrid mice. A smaller amount was prepared in the C5-deficient strains, A/J and B10-D2 old, so that antibody free of C5 could be transferred in appropriate experiments.

Chicken antirat serum (CARS) was prepared in two adult female chickens that had received five intravenous injections of cells pooled from rat spleens and lymph nodes. The injections consisted of $10^9$ cells and were given at weekly intervals. 1 wk after the last injection, the chickens were bled and the sera were pooled.

Rabbit antirat granulocyte serum was kindly provided by Dr. Charles G. Cochrane, Scripps Clinic and Research Foundation, La Jolla, Calif., and was prepared as described by Cochrane et al. (8). It had been absorbed in Dr. Cochrane’s laboratory with rat plasma, packed erythrocytes, and platelets to yield a reagent with a high degree of specificity for polymorphonuclear neutrophils.

The IgG fraction of RARS was isolated as described previously (2) and was digested with pepsin to obtain F(ab’)2 fragments according to the method of Nisonoff (9).

Serology.—Cytotoxic tests were carried out in vitro using mixtures of cells obtained from spleens and lymph nodes. Sera were tested in doubling dilutions using rabbit serum as a source of complement and dye exclusion as a criterion of viability. Details of this procedure are described in reference 10.

1 Abbreviations used in this paper: CARS, chicken antirat serum; COF, cobra venom factor; HAGG, heat-aggregated gamma globulin; MARS, mouse antirat serum; NCS, normal chicken serum; PMN, polymorphonuclear leukocytes; RAMLS, rabbit antimouse lymphocyte serum; RARS, rabbit antirat serum.
Hemolytic activity of mouse complement was measured by a specially devised microprocedure that allowed us to work with very small volumes of fresh mouse serum and to detect low levels of activity after attempts to depress complement activity in vivo. Rabbit erythrocytes at a concentration of $6 \times 10^6$/ml were sensitized with an equal volume of mouse (A/Sn) antirabbit erythrocyte serum diluted 1:600 in buffered saline. This antiserum was known to contain a relatively high concentration of antibodies that were resistant to treatment with 2-mercaptoethanol and that behaved in chromatographic and gel filtration analysis as 7S gamma globulins. This type of antibody is known to be essential for the activation of hemolytic properties of mouse complement (11). Doubling dilutions of fresh mouse sera were prepared in microtiter plates in volumes of 0.025 ml. 9 #i of each dilution was transferred to individual wells of microtest plates (Falcon Plastics, Los Angeles, Calif.), and to each well was added 1 #l of sensitized rabbit cells. The mixtures were incubated at 37°C for 45 min, and the plates were observed under the microscope. Where complete lysis had taken place, there were no cells visible. The end point, which occurred sharply, was taken as the dilution at which approximately 50% of the cells were visible.

Heat-aggregated gamma globulin (HAGG) was prepared from a commercial preparation of human globulin (Hyland Div., Travenal Laboratories, Inc., Costa Mesa, Calif.). The protein was separated from the glycine stabilizer by gel filtration (Sephadex G-50) and heated for 20 min at 63°C in a water bath. The final concentration of protein was 100 mg/ml.

The purified fraction of cobra venom used for inhibition of complement in vivo was kindly supplied by Dr. R. A. Prendergast, Department of Microbiology, Johns Hopkins School of Medicine, Baltimore, Md. It had been prepared from crude venom according to the procedure described by Ballow and Cochrane (12).

The nitrogen mustard used to depress polymorphonuclear leukocytes (PMN) in vivo was Mustargen (Merck, Sharpe & Dohme, West Point, Pa.).

**RESULTS**

Rat skin grafted onto untreated mice healed into place and behaved essentially as do allografts of mouse skin. However, the xenografts provoked more intense responses than do allografts and were, accordingly, rejected at an earlier time than the former. The median survival time for grafts placed on 39 mice was 7.8 days. When the recipients had been thymectomized and treated with RAMLS, the rat skin survived for much longer periods of time, in some cases for up to 3 mo. The median survival time for grafts on 79 treated animals was 31.6 days (Fig. 1). The data presented in this figure come from a series of experiments in which several lots of RAMLS of varying potency were employed, but there was in every case marked prolongation of graft survival on treated mice. When B10-D2 (new) mice that were bearing completely healthy grafts on day 14 were injected with MARS, intense inflammatory responses developed in the grafts of all of the injected animals within 1–3 h, and at 24 h there was frank necrosis in all but 1 of 36 grafts. This single graft recovered from the early inflammatory process and was later rejected through the action of the host’s own immune responses. Four grafts were completely destroyed by 24 h, and in the remainder there were varying degrees of epithelial damage ranging upward from 25%. Most of these latter grafts deteriorated rapidly, and 80% of all of the grafts in this experimental group were totally destroyed at 72 h. The remaining six grafts had, at this time, areas of viable epithelium amount-
DESTRUCTION OF SKIN GRAFTS BY HUMORAL ANTIBODY

The process of graft rejection as it occurs in mice of all of these experimental groups has been studied microscopically, and the results of these studies are reported elsewhere (2). The work described here is based on gross observations of the grafts, and as can be seen from Fig. 2, the effects of MARS are striking indeed and easily detected.

That the variations observed in grafts in response to injections of MARS are not attributable to the use of marginal doses of antiserum can be seen from the results of experiments in which injected mice were bled and their sera tested for residual activity in vivo and in vitro. These experiments were designed to show also that MARS was present in recipients in excess of that re-

Fig. 2. (a) A Lewis rat ear skin graft surviving in perfect condition on a thymectomized, ALS-treated mouse at 14 days. Ear skin is normally less pigmented than skin from the body wall and has fewer hair follicles. (b) This photograph shows the acute changes produced in the same graft as in (a) 48 h after its recipient had received 0.5 ml of a mouse antirat serum intra-peritoneally. Darker zones represent advanced necrosis in the inflamed and edematous graft. Changes of almost as severe a degree were present in this graft at 24 h. × 10-11.
quired for graft destruction so that the availability of antibody would not be a limiting factor in the studies on the roles of PMN and complement in the destructive process.

Eight mice bearing rat skin were injected i.p. with 1.0 ml of MARS that had a cytotoxic titer of 1:1024. 24 h later, when all of the grafts had been destroyed, the mice were bled and the sera from individual bleedings were tested for cytotoxic activity. Five of the sera had titers of 1:256, the other three had titers of 1:512. All of the sera were pooled and injected i.p. at a dose of 2 ml into two additional mice that had been grafted with rat skin. The following day both of the grafts were necrotic, and the sera of both animals were found to have cytotoxic titers of 1:128. It seems unlikely that any of our experimental results can be attributed to limiting amounts of antibody.

The involvement of complement in the destruction of rat skin xenografts that follows the injection of their hosts with antiserum was investigated through the use of (a) non-C-fixing antibodies or antibody fragments, (b) agents capable of depleting complement components transiently in vivo, and (c) mouse strains with genetically determined deficiencies of C5.

Properties of F(ab′)2 Fragments Prepared from RARS.—The immunoglobulin fragments obtained by pepsin digestion of an IgG preparation were concentrated to the volume of serum that served as starting material, and they were tested for specific toxicity and specific inhibitory properties in vitro and in vivo.

In tests carried out in vitro the F(ab′)2 fragments were found to have low levels of C-dependent toxicity for rat lymphocytes (Table I). This might be attributed to the presence of undigested molecules in the preparation, but the ability of this material even when diluted over 100-fold to inhibit the cytotoxicity of RARS from which it was derived argues against this interpretation. It seems more likely that F(ab′)2 obtained by pepsin digestion retains some ability to activate rabbit C, and this activity is detectable only when high concentrations of the fragments are employed.

| Dilution of F(ab′)2 | Cytotoxic titer |
|---------------------|----------------|
|                     | MARS           | RARS           |
| 1:1024              | 1:512          |                |
| 1:4                 | 1:4            | 1:4            |
| 1:4                 | 1:4            | 1:4            |
| 1:64                | 1:32           |                |

* F(ab′)2 fragments were prepared by pepsin digestion of IgG and reconstituted to the volume of serum from which the IgG was prepared. This preparation had a C-dependent cytotoxic titer of 1:16.
† Rat lymphoid cells were incubated with F(ab′)2 for 30 min at 37°C, washed twice with balanced salt solution, and then tested with MARS and RARS and rabbit C.
In the inhibitory tests carried out with F(ab')2 derived from RARS, the rat lymphoid cells were incubated with fragments and washed before incubation with dilutions of antirat serum and C. In these circumstances the cytotoxic properties of MARS were also blocked by the F(ab')2 (Table I).

When the fragments were injected into mice that had been grafted with rat skin, signs of mild inflammatory processes were seen in some of the grafts, but these invariably subsided and the grafts were actively rejected by their hosts more than a week later (Table II). If mice bearing rat skin grafts were injected with F(ab')2 and then 24 h later with IgG (RARS) or MARS, the grafts were unaffected by the second injections that, as seen in control animals, could be expected to induce inflammation in all of the grafts and to cause destruction of most of them.

### TABLE II

**The Effect of CARS or F(ab')2 from RARS on Xenografts of Rat Skin**

| Treatment i.p. | no. of mice (B10 D2 controle) | no. of grafts showing inflammation | no. of grafts destroyed |
|---------------|-----------------------------|----------------------------------|------------------------|
|               |                             | Severe* mild and transient†       |                        |
| IgG (RARS)    | 2                           | 2                                 | 2                      |
| F(ab')2 (RARS) | 6                           | 0                                 | 2 0                    |
| +             | 2                           | 0                                 | 2 0                    |
| IgG (RARS)    | 0                           | 0                                 | 0                      |
| MARS          | 12                          | 12                                | 10                     |
| F(ab')2 (RARS) | 0                           | 9                                 | 0                      |
| +             | 12                          | 0                                 | 0                      |
| CARS          | 14                          | 0                                 | 0                      |
| NCS†          | 5                           | 5                                 | 0 5                    |
| MARS          | 11                          | 0                                 | 1 0                    |

* Edema and erythema appeared within 1-2 h after administration of serum and increased in intensity for 4-6 h at which time there was evidence of interstitial hemorrhage. In all but two cases this was followed by necrosis and sloughing.

† Mild edema and erythema appeared at 4-8 h after administration of serum but were no longer detectable at 12 h. There was no hemorrhage or necrosis.

§ No viable epithelium detectable 24-48 h after administration of serum.

|| CARS, normal CS or F(ab')2 from RARS was injected, and the grafts were inspected periodically for 24 h after which the second material was injected. In the case of normal chicken serum (NCS) and CARS no reactions were observed during the first 24 h.
The Effect of Chicken Antirat Serum on Rat Skin Xenografts.—Antibodies produced in avian species interact poorly or not at all with complement components of mammalian plasma (13). CARS might, therefore, be expected to behave in our test system as did F(ab')₂ fragments prepared from RARS. In tests carried out in vitro, CARS was not toxic for rat lymphoid cells in the presence of rabbit C, guinea pig C, or mouse C. Furthermore, when rat lymphocytes were incubated with CARS for 30 min at 37°C and then washed twice with balanced salt solution and tested with MARS and rabbit C, the cytotoxicity of the latter reagents was markedly inhibited (Fig. 3).

After injections of CARS into mice bearing rat skin grafts, no signs of inflammation were detected in the grafts that survived for the same periods of time as grafts on uninjected mice (Table II). If the injections of CARS were followed by the administration of MARS 24 h later, the grafts were not damaged by the latter. In mice injected with normal chicken serum first and with MARS at 24 h, all of the grafts were destroyed 1–2 days later. Thus CARS that failed to activate mouse C in vitro not only fails to cause damage to the skin grafts in vivo but also blocks the action of MARS and is presumed, therefore, to have combined with graft antigens.

Destruction of Grafts on Hosts Depleted of Complement Components.—Initially, attempts to deplete mice of complement components were made by injecting the animals i.p. with HAGG. With this form of treatment it was difficult to reduce hemolytic activity of serum to less than 15–20% of control values, and it was not possible to maintain low levels for more than 12–15 h without inducing toxicity. By injecting 50 mg 1 h before and 4 h after the administration of MARS, we were able to reduce the level of C sufficiently to interfere with graft destruction without inducing any obvious side effects. In mice so treated there was a delay in the appearance of inflammation, and complete destruction

![Fig. 3. Inhibition of the C-dependent cytotoxic activity of MARS by CARS.](image-url)
of the grafts, which occurred in four out of six animals, was not observed until
days 3-5 after treatment with MARS (Table III). Since C levels had risen to
50% of control values by 24 h and were essentially normal at 36 h, it is not
surprising that these reactions were delayed rather than completely inhibited.

Table III also contains results of experiments in which mice were depleted
of complement components by the injection cobra venom factor (CoF), a
reagent which proved to be far more efficient than HAGG in preventing damage
to skin grafts after administration of MARS. The effect of CoF, given in various
doses, on the hemolytic activity of mouse C is shown in Fig. 4. The mice used in
this experiment were normal B10·D2 new females, and the CoF preparation

| Treatment: 1.0 ml MARS (i.p.) + | no. of mice (B10·D2 new) | First signs of inflammation | Rejection* (days) | Recovery† |
|--------------------------------|-------------------------|-----------------------------|------------------|-----------|
|                                |                         | <1 h 1-3 h 3-12 h           | 1 2 3-6          |           |
| HAGG§                           | 11                      | 9 2 — 7 2 — 2              |                  |           |
| CoF (0.1 ml) 1 h before MARS    | 6                       | — 3 3 — 4 1 2 1           |                  |           |
| CoF (0.3 ml) 1 h before MARS    | 8                       | — 2 6 6 1 1 —             |                  |           |
| CoF 0.3 ml 1 h before MARS      | 4                       | — — 4 — — — 4             |                  |           |
| CoF 0.1 ml 18 h after MARS      |                         |                            |                  |           |

* Rejection was taken as the point at which viable epithelium was no longer detected on
gross inspection.

† These grafts showed signs of inflammation of varying degrees of intensity after the in-
jection of MARS, but they recovered fully normal appearances and were actively rejected
2-3 wk later.

§ 50 mg of heat-aggregated human gamma globulin was injected i.p. 1 h before and 4 h
after MARS.

A dose of 0.3 ml suppressed all hemolytic activity of serum for at least 24 h,
and at 48 h there was about 30% recovery. Mice bearing skin grafts were in-
jected with either 0.1 or 0.3 ml 1 h before receiving MARS. There was a delay
in the appearance of inflammation in all of the grafts, but the pattern of de-
struction of these grafts was essentially the same as that seen in control mice
(Table III). Four mice were injected with 0.3 ml of CoF 1 h before MARS was
given and again with 0.1 ml 18 h after the antiserum had been injected. Signs
of inflammation appeared in all of the grafts, but the reactions were late in
developing and were milder than those seen in control mice. Furthermore, all
of these grafts recovered and were actively rejected 2-3 wk later. Thus CoF
can interfere with the chain of reactions initiated by MARS and leading to
DESTRUCTION OF SKIN GRAFTS BY HUMORAL ANTIBODY

A. C3

B. C4

C. C5

D. C6

E. C7

F. C8

G. C9

H. C1

I. C2

J. C1r

K. C1s

L. C1c

M. C1s

N. C1c

O. C1r

P. C1s

Q. C1c

R. C1r

S. C1s

T. C1c

U. C1r

V. C1s

W. C1c

X. C1r

Y. C1s

Z. C1c

Fig. 4. Inhibition of the hemolytic activity of mouse C after the injection of CoF.

graft destruction, but the amounts required for complete protection of the grafts were higher than might be anticipated on the basis of the measurements of hemolytic C. This is probably due to a requirement for higher concentrations of C3 for hemolysis than for the C-dependent reactions involved in graft damage. Since the role of C seems to lie in its ability to attract leukocytes and since very low levels of PMN suffice to cause graft damage (see below), this explanation seems plausible.

Antibody-Mediated Destruction of Rat Skin Grafted onto C5-Deficient Mice.—The importance of C5 in antiserum-mediated destruction of skin grafts was studied by comparing the effects of MARS on grafts placed on B10-D2 new (C5+) and B10-D2 old (C5−) mice. Antiserum was administered in doses ranging from 1.0 to 0.05 ml in the case of B10-D2 new mice and from 1.0 to 0.1 ml in B10-D2 old animals. The results of this experiment are presented in Table IV A. At a dose of 1.0 ml there were inflammatory processes in all of the grafts of both lines of mice, but there were important differences in their overall responses to this treatment. In C5(+) recipients signs of inflammation appeared earlier and hemorrhage and necrosis were more commonly observed; all but 1 of 26 grafts were completely destroyed by 3 days and 16 of these had no viable epithelium at 24 h. In C5(−) mice 12 of 20 grafts were destroyed by treatment with antiserum; one of these was sloughed on day 2 and the other 11 on days 4 and 5 after treatment with MARS.

At lower doses of MARS the differences in the responses of the two strains of mice were even more striking. Only 1 of 12 grafts on C5(−) mice injected with 0.1-0.5 ml of serum was destroyed; whereas, all of the 13 grafts on C5(+) mice treated with similar doses were destroyed, and 3 of 5 grafts on mice treated with 0.05 ml of MARS were also destroyed. Thus a far greater quantity of antiserum is required to cause graft destruction in B10-D2 old mice, and the tempo of the destructive process is consistently slower in these recipients than in B10-D2 new mice.
TABLE IV A

The Influence of C5 on the Pattern of Antiserum-Mediated Destruction of Rat Skin Graft

| No. of mice | C5 | MARS (ml) | First signs of inflammation | Rejection (days): | Recovery§ | No reaction |
|-------------|----|-----------|-----------------------------|------------------|-----------|------------|
|             |    |           | <1 h | 1-3 h | 3-12 h | 1 | 2 | 3-5 | |
| 26          | +  | 1.0       | 20   | 5    | 1     | 16 | 7 | 2       | 1       | —         |
| 6           | +  | 0.5       | 6    | —    | —     | 1  | 4 | 1       | —       | —         |
| 7           | +  | 0.1       | 6    | 1    | —     | 1  | 5 | 1       | —       | —         |
| 5           | +  | 0.05      | 3    | 2    | —     | 1  | 1 | 1       | 2       | —         |
| 20         |    | 1.0       | 1    | 5    | 14    | —  | 1 | 11      | 8       | —         |
| 3           |    | 0.5       | —    | 1    | 2     | —  | — | 1       | 2       | —         |
| 5           |    | 0.25      | —    | —    | 5     | —  | — | —       | 5       | —         |
| 4           |    | 0.1       | —    | —    | 1     | —  | — | —       | 1       | 3         |

* Rat skin was grafted to female mice of the strains B10.D2 new (C5+) or B10-D2 old (C5−) and MARS was injected i.p. at the doses indicated.
† Rejection was taken as the point at which viable epithelium was no longer detected on gross inspection.
§ These grafts showed signs of inflammation of varying degrees of intensity after the injection of MARS, but they recovered fully normal appearances and were actively rejected 2–5 wk later.
|| One-half of the mice in each of these two groups received MARS that had been prepared in A/J or B10-D2 old mice so that no C5 was introduced with the antibody. There were no differences in the reaction of mice receiving either type of MARS.

The C5 is responsible for these differences can be seen from the results of experiments in which fresh serum from C5(+) mice was injected in B10-D2 old graft recipients along with MARS (Table IV B). In five mice treated with 0.4 ml of fresh normal serum mixed with only 0.1 ml of MARS, the grafts showed early, intense inflammation, and four of the grafts were completely destroyed within 48 h. The remaining graft had about 50% of its epithelium destroyed at this time, but the rest of the graft was not damaged until several days later when many of the control grafts were being actively rejected. The activity of the fresh normal serum was destroyed when it was heated to 56°C for 30 min.

The results of this last experiment might be construed to mean that the data presented in Table IV A come from responses to variations in the dose of C5 contained in the MARS and that in the absence of C5 no tissue damage can occur. However, as indicated in Table IV A, half of the mice that were injected with 1.0 ml of MARS received serum that had been prepared in C5-deficient animals, and the reactions observed in their grafts were not distinguishable from those observed in grafts on mice injected with MARS prepared in B6AF1 mice. Furthermore in a study of MARS prepared in various inbred strains of
The Influence of C5 on the Pattern of Antiserum-Mediated Destruction of Rat Skin Grafts

| No. of mice | MARS + (0.1 ml) | First signs of inflammation | Rejection (days) | Recovery |
|-------------|-----------------|-----------------------------|-----------------|---------|
|             |                 | <1 h | 1-3 h | 3-12 h | 1 | 2 | 3-5 |         |
| 5           | 0.4 ml saline   | 0    | 2     | 3     |   |   |     | 5       |
| 5           | 0.4 ml C5+ serum| 0    | 4     | 1     | 0 | 1 | 3   | 1       |
| 5           | 0.4 ml C5+ serum (heated 56°C for 30 min) | 0    | 2     | 3     |   |   |     | 5       |

* Rat skin was grafted to B10-D2 old male mice that lack C5.

‡ All of the mice were injected i.p. with a mixture containing 0.1 ml of MARS and 0.4 ml of saline or normal mouse serum as indicated.

§ Rejection was taken as the point at which viable epithelium was no longer detected on gross inspection.

|| These grafts showed signs of inflammation of varying degrees of intensity after the injection of MARS, but they recovered fully normal appearances and were actively rejected 2-7 wk later.

mice the ability of C5-deficient antisera to mediate graft destruction in C5(−) recipients was observed in large numbers of mice.²

The survival time of rat skin grafted onto nonsuppressed mice lacking C5 was the same as that for grafts placed on C5+ animals, and no significant differences were seen in the survival times of grafts placed on mice of these two sublines when their immune responses were suppressed by thymectomy and treatment with RAMLS.

The Role of PMN in Antiserum-Mediated Destruction of Skin Grafts.—The numbers of circulating PMN in mice bearing rat skin grafts were decreased by treatment with nitrogen mustard or by the administration of rabbit antirat PMN serum. The action of nitrogen mustard is far less specific than that of antiserum, and it was found difficult with this reagent to maintain the counts of circulating PMN at low levels without causing toxic side effects in mice. However, a regimen of 0.05 mg of Mustargen given 3 days before MARS and 0.025 mg given 1 day before MARS resulted in a reduction in PMN counts to 40-80 cells/mm³ at the time of administration of the antiserum, without inducing untoward reactions and without lowering the levels of hemolytic C. In mice so treated the MARS had little influence on the fate of the skin grafts (Table V). In one of seven recipients there was late and transient inflammation in the graft; no effects were observed in the grafts on the other six mice.

Two mice that had received rat skin grafts were treated with rabbit antirat PMN serum at a dose of 0.3 ml on days 3, 2, and 1 before administration of MARS. The numbers of PMN in the peripheral blood of these two animals were 220 and 230/mm³; platelet counts and C levels were normal. There was

² Jooste, S. V. 1973. Ph.D. Dissertation. University of Pretoria, South Africa.
The Role of MN in Antiserum-Mediated Destruction of Rat Skin Grafted onto Mice

| Treatment: 1.0 ml MARS + N-mustard§ Anti-PMN serum | No. of mice (B10 D2 new) | Total WBC/mm³ | PMN/mm³ | Inflammation | Rejection* Recovery† |
|---|---|---|---|---|---|
| — | 11 | 1.5-2.0 \times 10⁴ | 4-5 \times 10³ | 9 | 2 | 0 | 9 | 2 |
| N-mustard§ | 7 | 1-2 \times 10⁴ | 40-80 | — | 1 | 7 | — | 1 |
| Anti-PMN serum | 2 | 2.2 \times 10⁴ | 220-230 | — | 1 | 1 | — | 1 |

* Rejection, taken as the point at which viable epithelium was no longer detected on gross inspection, occurred 24-48 h after administration of MARS in these mice.

† These grafts showed signs of inflammation of varying degrees of intensity after the injections of MARS, but they recovered fully normal appearances and were actively rejected 2-3 wk later.

§ 0.05 mg 3 days before MARS and 0.025 mg 1 day before MARS.

II 0.3 ml rabbit antirat PMN serum on days 3, 2, and 1 before MARS.

DISCUSSION

The process of tissue destruction that we have described here is, undoubtedly, the outcome of a complex series of events that are initiated by the combination of antibodies with graft antigens and that involve the generation of a variety of substances that affect the tone of small blood vessels and the integrity of their walls. These substances are in many instances the end products of multi-stepped, sequential reactions, and they interact among themselves in ways that are not well understood. Our data shed no light on the nature or direct mode of action of these effector substances, but they do show that complement and polymorphonuclear leukocytes play essential roles in the development or expression of them.

With respect to complement, our data provide several lines of evidence that some components are essential for the production of graft damage that follows the injection of antibodies. Neither CARS nor F(ab')₂ derived from RARS serve as efficient activators of mouse C, and neither caused destruction of grafts though it could be inferred from their inhibitory properties in vivo that they had combined with graft antigens. Secondly, depletion of complement components in vivo with CoF or heat-aggregated gamma globulin prevented or delayed the occurrence of tissue destruction that routinely follows the injection of MARS. Lastly, our experiments with C5-deficient mice implicate components
of C as mediators of graft damage and help also to clarify the roles of some individual components in this process. Rat skin grafted onto either B10-D2 new (C5+) or B10-D2 old (C5-) mice was destroyed by injections of MARS, but in the case of the former strain the process was initiated much sooner, it developed more rapidly, and required less antibody. While C5 is not essential for the production of tissue damage in this process, it, or some components that act later in the complement sequence, can obviously contribute to the tissue destruction, and in a more efficient way than earlier acting components.

That antiserum can cause destruction of grafts in the absence of C5 shows that direct lysis of graft cells by C is not essential in this process. Indeed, C-mediated lysis does not seem to occur to an appreciable extent even in B10-D2 new mice since tissue damage was not detected when C levels were normal, but the numbers of PMN were greatly reduced. The requirement for PMN suggests that the role of C is to bring the leukocytes to the sites at which antibodies have become bound to antigens in the graft. This is an attractive suggestion since it fits well with all of our observations as well as with known properties of chemotactic substances derived from C. There are at least two stages in the sequence of C reactions at which substances chemotactic for PMN are generated. After the reaction of C3 with C142 there appears a derivative of C3, probably C3a, that has been shown to attract PMN in vitro (14). Further on in the sequence the reaction of C5 with C1423 is followed by the appearance of a powerful chemotactic substance, the composition of which is not precisely known. It has been claimed that the activity resides in a split product of C5, (C5a), as well as in a trimolecular complex C567 (15, 16). In either case there is agreement on the importance of C5, and in a recent study it has been shown that mice lacking C5 do not mobilize PMN in response to inflammatory stimuli involving C nearly as efficiently as C5+ mice do (17). The difference between the responses of the two types of animals was especially marked during the first 24 h after application of the stimuli. These observations are consistent with the demonstration that the chemotactic activity of substances containing or derived from C5 is much greater than that of C3 derivatives (18).

On the basis of this discussion we can offer a plausible construction of the events leading up to damage of the skin grafts in our experimental system. Antibodies react with antigens in the graft, probably those present on the surface of vascular endothelial cells. These immune complexes activate C1 initiating a series of events the precise nature of which depends on the availability of individual components of the complement system and the level of circulating PMN as well as other factors not directly implicated in the present study but known to be of importance in the production of tissue damage by immune reactions. In mice with normally functioning components of C, chemotactic substances are generated from both C3 and C5, and PMN are rapidly brought into the vascular bed of the graft that becomes the site of an intense inflammatory process. Damage to the graft then becomes a consequence of the action of the
PMN as well as of a variety of substances that appear at sites of inflammation. In mice that lack C5 the attraction of PMN into the graft depends largely on the action of C3a, though immune adherence may also participate in this process. Cells are mobilized slowly; the inflammatory process develops only gradually and often subsides before significant tissue damage occurs. CoF depletes animals of C3 and later-acting components thus impeding the generation of chemotactic agents and preventing damage to the graft at least until the levels of relevant components are restored. It is not clear which components of C are depleted after injections of aggregated gamma globulin, but it can be inferred that the development of chemotactic factors is retarded and that damage to grafts in mice treated first with HAGG and then with MARS is delayed or absent.

The participation of polymorphonuclear leukocytes in the type of graft destruction described here was initially suggested by the results of microscopic studies of fixed tissues (1). The essential role of these cells in the process is indicated in the present study by the results of experiments in which either nitrogen mustard or specific antiserum was used to depress the numbers of circulating PMN. When the numbers of the cells were brought to very low levels, tissue damage was prevented even though C was present in normal concentrations. The destruction of vascularized grafts of skin by humoral antibodies is thus an additional example of immune damage to tissues in which PMN seem to be of central importance.

The mechanisms through which leukocytes cause destruction of the grafts are not illuminated by the results of our present studies, but it seems reasonable to suppose that they are similar to those involved in Arthus reactions and other forms of vasculitis and immune damage triggered by the combination of antigens with antibodies in vivo (19). Further experimentation obviously will be needed to clarify this matter.

We have emphasized the importance of antigens on endothelial cells for two reasons. First, the time that lapses between intravenous administration of antiserum and the earliest signs of inflammation in the graft is often less than 10 min. It seems unlikely that antibody could have left the vascular bed and triggered an inflammatory response in this short period of time. Secondly, our microscope observations suggest that vascular breakdown followed by hemorrhage and thrombosis precedes and probably accounts for damage to non-vascular elements of the grafts. Indeed, high concentrations of antibody may reach the latter only when the vasculature is badly damaged. That we have seen intense edema in some grafts that were not destroyed after administration of antiserum shows that when antibodies do have access to cells lying outside the blood vessels, they do not always cause detectable damage to the graft; but this issue can probably be resolved only through the use of grafts of pure epidermal cells.

While essential roles for C and PMN have been established for the destruc-
DESTRUCTION OF SKIN GRAFTS BY HUMORAL ANTIBODY

tion of skin grafts by antisera in our experimental system, it seems unlikely that they will turn out to be the only factors whose participation is obligatory, and there will almost certainly be ancillary substances that contribute to the development of the inflammatory process accompanying destruction but that may not be indispensable to the overall reaction. It should be possible, through further studies, to delineate the roles of all of these effector substances and to provide a clearer picture of the ways in which humoral antibodies can bring about the destruction of vascularized grafts.

SUMMARY

A study has been made of the roles played by complement and polymorphonuclear leukocytes (PMN) in the acute destruction of xenografts of rat skin that follows injection of their hosts with antisera specifically reactive with graft antigens. The rat skin was grafted onto mice whose immune responses were suppressed by removal of the thymus and a brief course of treatment with rabbit antimouse lymphocyte serum. At about 2 wk after grafting the mice were injected intravenously or intraperitoneally with mouse antirat serum (MARS). This time interval was chosen because it avoided the complications that might be associated with either the process of healing in or with incipient rejection. Signs of graft damage were evident as early as 10 min after the injection of MARS, and in most animals so injected the grafts were completely destroyed within 24-48 h.

The role of complement (C) in this acute destructive process is indicated by the results of three lines of experimentation. (a) Non-C-fixing antibodies or antibody fragments failed to cause damage to the grafts. Indeed, both chicken antirat serum and F(ab')2 fragments from rabbit antirat serum completely protected the grafts against the effects of MARS that was administered 24 h later. (b) When mice were depleted of hemolytic C by treatment with cobra venom factor or heat-aggregated gamma globulin, the damage caused by MARS was greatly reduced or completely inhibited. (c) In mice with a genetically determined absence of C5 much greater quantities of MARS were required to cause graft damage; the tempo of the destructive process was consistently slower; and a greater number of grafts recovered from the initial inflammatory process than was the case for animals with an intact comple-

ment system.

The participation of PMN in serum-mediated destruction of grafts was initially suggested by the results of microscope examination of fixed tissues. The essential role of these cells in the process is indicated by the failure of MARS to cause tissue damage in mice whose circulating PMN have been reduced to very low levels by treatment with nitrogen mustard or more specifically with an anti-PMN serum.

The absence of tissue damage when circulating PMN are reduced but C
levels are normal suggests that C-mediated cytolysis is unimportant in graft destruction and that the role of C lies in its ability to generate chemotactic factors. The latter may then attract the PMN that provide mediators of tissue damage.

REFERENCES
1. Baldamus, C. A., H. J. Winn, and P. S. Russell. 1970. Acute rejection of skin xenografts in the mouse after passive transfer of humoral antibody. Fed. Proc. 29:785.
2. Baldamus, C. A., I. F. C. McKenzie, H. J. Winn, and P. S. Russell. 1972. Acute destruction by humoral antibody of rat skin grafted to mice. J. Immunol. In press.
3. Cochrane, C. G. 1965. The Arthus reaction. In The Inflammatory Process. B. W. Zweifach, L. Grant, and R. T. McCluskey, editors. Academic Press, Inc., New York. 613.
4. Humphrey, J. H., and R. G. White. 1970. Immunology for Students of Medicine. F. A. Davis, Co., Philadelphia, Pa.
5. Cochrane, C. G. 1967. Mediators of Arthus and related reactions. Prog. Allergy. 11:1.
6. Snell, G. D., and J. H. Stimpfling. 1965. Genetics of tissue transplantation. In Biology of the Laboratory Mouse. E. L. Green, editor. McGraw-Hill Book Co., New York. 457.
7. Alper, C. A., and F. S. Rosen. 1971. Genetic aspects of the complement system. Adv. Immunol. 14:251.
8. Cochrane, C. G., E. R. Unanue, and F. J. Dixon. 1965. A role of polymorphonuclear leukocytes and complement in nephrotoxic nephritis. J. Exp. Med. 122:99.
9. Nisonoff, A. 1964. Enzymatic degradation of rabbit gamma globulin and antibody and chromatography of digestion products. Methods Med. Res. 10:134.
10. Sachs, D. H., H. J. Winn, and P. S. Russell. 1971. The immunologic response to xenografts. Recognition of mouse H-2 histocompatibility antigens by the rat. J. Immunol. 107:481.
11. Winn, H. J. 1965. Effects of complement on sensitized nucleated cells. Complement, Ciba Found. Symp.
12. Ballow, M., and C. G. Cochrane, 1969. Two anticomplementary factors in cobra venom: hemolysis of guinea pig erythrocytes by one of them. J. Immunol. 108:944.
13. Rose, M. E., and E. Orlans. 1962. Fowl antibody. III. Its haemolytic activity with complements of various species and some properties of fowl complement. Immunology. 5:533.
14. Ward, P. A. 1967. A plasmin-split fragment of C3 as a new chemotactic factor. J. Exp. Med. 126:189.
15. Ward, P. A., C. G. Cochrane, and H. J. Müller-Eberhard. 1966. Further studies on the chemotactic factor of complement and its formation in vivo. Immunology. 11:141.
16. Shin, H. S., R. Snyderman, E. Friedman, A. Mellors, and M. M. Mayer. 1968.
Chemotactic and anaphylatoxic fragment cleared from the fifth component of guinea pig complement. *Science (Wash. D. C.).* **162**:361.

17. Snyderman, R., J. K. Phillips, and S. E. Mergenhagen. 1971. Biological activity of complement in vivo. Role of C5 in the accumulation of polymorphonuclear leukocytes in inflammatory exudates. *J. Exp. Med.* **134**:1131.

18. Mayer, M. M., H. S. Shin, and R. Snyderman. 1969. Chemical and biological studies of the fragmentation of complement components C2, C3 and C5. *In Complement Symposium, Mainz, West Germany.* (Abstr.)

19. Cochrane, C. G. 1969. Immunologic tissue injury mediated by neutrophilic leukocytes. *Ad. Immunol.* **9**:97.