Antibody Response to Skin Allografts in Mice (34697)

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(Introduced by J. L. Fahey)

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Circulating antibodies following skin allograft rejection have been demonstrated by several investigators (1–8). There is little evidence, however, that these antibodies are important factors in the mediation of graft rejection (9). The dose–response and temporal relationships between allograft rejection and antibody formation have received relatively little attention (3, 10). The experiments reported here were designed to study these relationships with a sensitive isotopic antiglobulin technique (11). Some grafts were removed prior to rejection to determine the length of time a skin graft had to be present on an animal to stimulate antibody formation and to determine the effect of the presence of the graft on antibody level.

Materials and Methods. Mice of three inbred strains were tested. B10.D2/JaX (H2-d) mice were tested with C57Bl/10 (H2-b) skin grafts, which differ only at the strong H-2 histocompatibility locus. BALB/cAn (H2-d) mice were tested with C57Bl/6N (H2-b) skin grafts, which differ at both H-2 and non-H-2 histocompatibility loci. Single, orthotopic skin grafts (1 cm^2) were made on the back and were held in place with 9-mm Autoclips (Clay-Adams, New York). After grafting, all animals were housed separately. They were divided into groups of 5–10 animals, and grafts were either left intact or were removed at days 2, 3, 5, 7. and 9. The clips were removed from the remaining grafts at day 7. The grafts were observed daily for visible and tactile signs of rejection.

The mice were bled from the retro-orbital sinus before grafting and at 3–4-day intervals thereafter. Aliquots of serum were then stored at —20° until tested. The isotopic antiglobulin technique (11) was used to test the sera for antibody activity. All of the sera from an individual animal were tested at the same time. All sera were tested in duplicate or triplicate. The C57Bl/6 ascitic leukosis (EL-4) was used for the target cells in all tests. Target cells (5×10^5 to 1×10^6 in 0.1 ml) were incubated with 0.1 ml of 1:5 dilution of serum for 30 min at room temperature in 10 × 75-mm siliconized test tubes. They were then washed seven times with 1 ml of Veronal buffered saline, pH 7.4, containing 10% fetal bovine serum (VBS/10% FBS). The cells were then incubated with 2 μl of ^{125}I-antiglobulin for 15 min at 0°. The cells were again washed five times and then transferred to plastic counting tubes. Cells were counted for ^{125}I gamma emissions.

A considerable range of antiglobulin uptake (^{125}I cpm) was noted when the results of the pregraft sera of the individual animals were compared. Testing of serial serum samples from the same unimmunized animal, however, yielded ^{125}I cpm which varied by only 5%. To determine the time of appearance of antibody activity, the results obtained with each animal's sera after grafting were compared with its own pregraft level. Geometric means of the test values within an experimental group were also calculated.

Specificity of the rise in ^{125}I cpm detected in the postgraft sera was demonstrated by also testing the sera against syngeneic spleen target cells. No increase over the pregraft levels was noted.

Some of the sera were also tested for cyto-

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toxic antibodies. One-tenth ml of serial twofold dilutions of sera was incubated with 0.1 ml of target cells (10^6 cells/ml) for 30 min at 37°. One-tenth ml of undiluted guinea pig serum, as a source of complement, was then added to each tube and the incubation was continued for another 30 min at 37°. Three-tenths ml of 0.2% trypan blue in saline was then added and the percentage of stained cells was determined.

Results. Adult B10.D2 mice rejected first set C57Bl/10 skin grafts in 9–12 days. A substantial increase in antiglobulin uptake was first detected in the serum of one animal at day 6 and in the sera of the other animals by day 9–10. Figure 1 summarizes the results with a plot of the geometric means of each day's results. The antibody levels rose until day 15 and then reached a plateau of three times the pregraft control level.

Similar results were obtained with the C57Bl/6→BALB/c combination. The skin grafts were rejected in 10–14 days. Antibody activity was detected in the sera at 10 days, the level rose until day 18, and was still present at day 33.

No antibody activity was detected in the groups in which the grafts were removed after two days. When the C57Bl/10 grafts were removed at day 3, only five of the seven

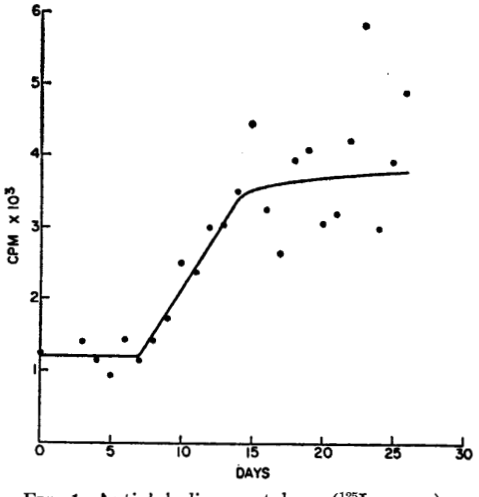

FIG. 1. Antiglobulin uptake (^{125}I cpm) with B10.D2 sera after grafting with C57Bl/10 skin. The points represent the geometric means of the results obtained with the individual sera on each day.

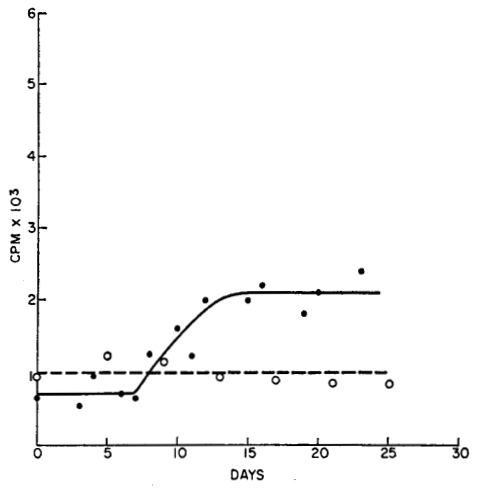

FIG. 2. Antiglobulin uptake with B10.D2 sera; C57Bl/10 skin grafts removed at day 3. Five of seven animals responded with detectable antibody formation (●) (geometric means of these results). The sera of two mice showed no increase in antiglobulin uptake (○).

B10.D2 mice responded with detectable antibody formation (Fig. 2). The initial appearance occurred on day 8–10, and only low levels of antibody were detected. When grafts were removed at day 5, again only five of seven animals responded with an increase in ^{125}I cpm (Fig. 3), with a time course similar to the other groups. Three of these responding animals had levels similar to the groups

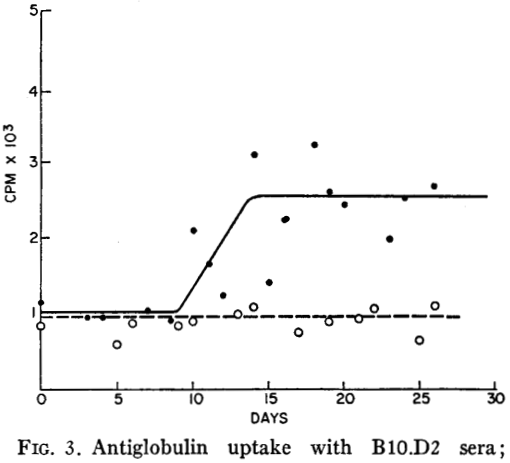

FIG. 3. Antiglobulin uptake with B10.D2 sera; C57Bl/10 skin grafts removed at day 5. Geometric means for the 5 responding animals (●) and the 2 nonresponders (○) are plotted separately.

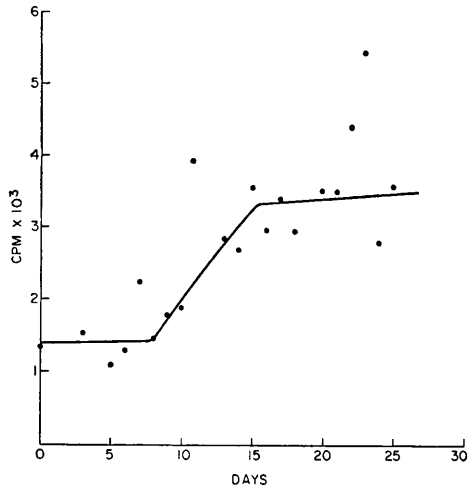

FIG. 4. Antiglobulin uptake with B10.D2 sera; skin grafts removed at day 7.

with grafts left intact, while the other two produced only low levels of antibody. When grafts were removed at 7 days, all of the animals responded in the same fashion as the intact group, and the levels reached were very similar (Fig. 4).

The antibody response to second set skin grafts was also studied. In one experiment, pooled sera of adult B10.D2 mice grafted with C57Bl/10 skin showed 17,500 ^{125}I cpm at 35 days after first set grafting, 10 times the antiglobulin uptake found with the base line normal B10.D2 serum (Fig. 5). Two days after a second C57Bl/10 graft was put on, the levels decreased (12,500 cpm) and then gradually increased to 37,500 cpm, 5 days after the second set grafts were completely rejected.

Antibodies were detected in some sera that were undetectable by the cytotoxic antibody assay. In one experiment, 20 BALB/c mice were grafted with C57Bl/6 skin. Five mice were bled each day and the sera were pooled. The sera were tested by both the isotopic antiglobulin technique and by the cytotoxicity technique. As was found in the previous experiments, the ^{125}I cpm rose at days 9–10 to over three times the control level. No cytotoxic antibodies were detected in these sera.

Discussion. The presence of circulating antibodies in serum after skin allografts has been well established. The temporal relationship of these antibodies to graft destruction and their role in the rejection process have not been as clearly demonstrated. In most studies, antibodies were first detected several days after the grafts were completely rejected (1, 2, 5, 7, 12, 13). This apparent lack of close temporal relationship has been cited as evidence that antibodies do not play an important role in graft rejection (13). However, other explanations have been offered for this lack of correlation: (a) insufficient sensitivity of serologic assays employed (9), (b) different molecular species of antibodies which may cancel each other's effects (13, 14), and (c) *in vivo* absorption of antibodies by the graft (7, 15, 16).

With a sensitive hemolytic plaque assay, it has been reported that antibody production occurs as early as 2–3 days after skin allografting (3). In some cases, however, there was a poor temporal correlation between the peak antibody response and graft rejection.

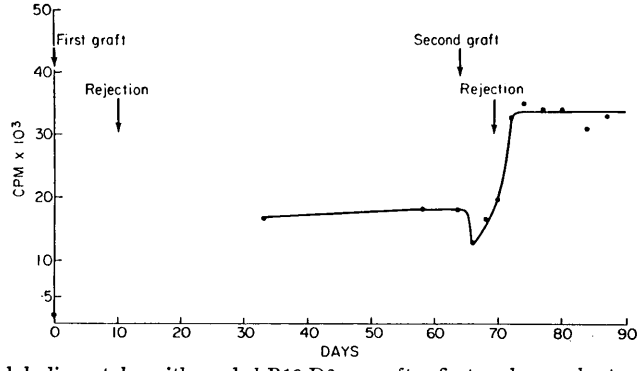

FIG. 5. Antiglobulin uptake with pooled B10.D2 sera after first and second set grafts.

Since some histocompatibility antigens are not readily detected on erythrocytes (17), the applicability of this assay may be somewhat limited.

The present study was designed to further investigate the onset of production of alloantibodies and their role in allograft rejection. The assay method used, an isotopic antiglobulin technique, has been shown to be more sensitive than the cytotoxicity method and has the potential of detecting antibodies which lack the functional characteristics needed for cell lysis or agglutination (11). Antibodies were first detected here shortly before or at the onset of graft rejection and peaked after rejection was complete. A possible explanation for this somewhat late time course was that the antibodies were actually being formed earlier, but were absorbed *in vivo* by the grafts (15, 16). To explore this possibility, grafts of some animals were removed prior to rejection. When the grafts were removed before day 7, some of the animals produced no detectable antibodies and others had diminished responses. Antibodies were not detected earlier than when the grafts were left intact. No evidence for *in vivo* absorption of antibodies by the first set skin grafts was obtained. The data suggested that continued presence of the alloantigens was important for maximal antibody production. Similar findings have been reported for hemagglutinin production (2, 15).

The antiglobulin reagent used in the present study reacted mainly with mouse IγG (γ_1, γ_{2a}, γ_{2b}) and only slightly with mouse IgM (11). It is quite possible that a specific anti-IgM reagent would detect earlier antibody response and testing of the sera with such a reagent will be performed in future experiments.

When a second set C57Bl/10 graft was put on B10.D2 mice, there was a transient decrease in antibody levels. This result was suggestive of *in vivo* absorption of alloantibody by the graft. Since serial bleedings were performed on the animals, it was also possible that this produced the antibody depletion. However, only small volumes of blood were removed, and bleeding to the same extent in other experiments did not result in lowered levels of antibody. This suggestion of *in vivo* antibody absorption by a second set graft and the high levels of antibody reached after the accelerated rejection indicate that antibodies may play a more important role in second set allograft rejection.

Summary. Antibody production in response to skin allografts in mice was studied with an isotopic antiglobulin technique. This technique has been shown to be sensitive and capable of detecting antibodies not demonstrable by the cytotoxicity technique. Antibodies were first found in most animals at 9–10 days after grafting, at the time of onset of graft rejection. Experiments were performed to determine whether antibodies were being formed earlier, but were absorbed *in vivo* by the grafts. When primary skin grafts were removed before rejection, antibodies were not detected earlier. Lower levels of antibodies occurred, indicating that continued presence of the alloantigens were important for maximal antibody production.

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