Measurement of Serum Complement During Homograft Rejection in Man and Rat.* (29129)

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Complement (C′) is required for immune cytotoxic phenomena such as immune hemolysis(1,2), the serum bactericidal reaction (3), or the lysis of certain tumor cells(4,5). The specificity of the cytotoxic reaction is determined by antibody, whereas complement is a non-specific group of serum factors which destroys cells previously sensitized by interaction with antibody. Complement may also be involved in immune injury of the Arthus type(6) which depends on the physical presence of an antigen-antibody aggregate followed by the deposition of platelets, leukocytes, and thrombin. Whether complement is involved in homograft rejection is unclear, but it might participate either as an adjunct to humoral antibody or in association with some specific cellular mechanism or both.

Studies on the effect of renal homografts in the dog(7,8) and man(9) have failed to demonstrate a fall in serum complement during the rejection process. Instead, the serum complement titer has risen, presumably due to surgical trauma(7), since the titer also increased following autografts. A variety of inflammatory processes elicit a rise in serum complement(10) and it seems possible that a non-specific increase with inflammation might mask concomitant complement fixation by immunologic events associated with the rejection process.

Fife, Hook, and Muschel(11) observed a reduction of Serum C′ in rats suffering from runt disease. This reduction may be immunologic in nature or be due to an inhibition of production of C′ in association with the complex pathological changes which occur in this disease(12).

In addition to the theoretic interest surrounding the question of the participation of complement in homograft rejection the subject gains additional importance since there is a need for methods capable of detecting incipient homograft rejection and of differentiating such an event from other causes of graft malfunction in clinical situations.

Accordingly serum C′ levels were studied in rats following first and second set skin grafts, white graft reactions, and injection of suspensions of homologous cells. The cell suspensions were used so as to minimize the non-specific influence on serum C′ of the surgical trauma attendant on skin grafting. Four patients with renal homografts were also followed by measuring both whole serum complement and the titer of the second component of human complement ($C'_2{}^{hu}$). The titer of the latter appears to be a more sensitive index of *in vivo* antigen-antibody interaction than the whole complement determination (13).

Materials and methods. Adult rats of both sexes, belonging to 2 highly inbred strains were used. Animals of the Fisher strain weighing from 180 to 220 g were used as recipients in all experiments, while donor material was obtained from adult Wistar (WR) rats.§ Full thickness grafts of skin were obtained from the flank of a recently sacrificed WR donor under aseptic conditions. Grafts were roughly rectangular and measured approximately 2 × 2 cm. They were placed in open style upon the exposed surface of the panniculus carnosus on the lateral thoracic wall and were held in place

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§ These animals were originally obtained from the Childrens Hospital, Boston, through the courtesy of Dr. Jacob Furth, and have been maintained by pedigreed sibling matings during the succeeding 4 years. Test skin grafts exchanged amongst randomly selected members of each strain uniformly survived indistinguishably from autografts for the full period observed (100 days).

by a light plaster jacket surmounting a fitted vaseline gauze square, according to the method of Billingham and Medawar(14).

Blood samples were collected by venipuncture from human recipients and by retro-orbital venous puncture from the recipient rats. The blood was clotted at room temperature for 30 (man) or 60 (rat) minutes, centrifuged at 4°C, and the supernatant stored in aliquots at —70°C. The whole complement titer (CH_{50}) in rat or human sera was measured as described by Mayer(15); the experimental error on duplicate samples was less than 5%. All specimens obtained from an individual recipient were assayed on the same day, when feasible. The same batch of sensitized cells (EA) was used when the number of samples was so great that titration required several days. The second component of human complement ($C'_2{}^{hu}$) in whole human serum was measured by its specific interaction with a cellular intermediate prepared with guinea pig complement, the $EAC'la^{gp},4^{gp}$ cell. In this interaction the conversion of $SAC'la^{gp},4^{gp}$ sites to $SAC'la^{gp},4^{gp}$, 2^{hu} sites is stoichometrically related to the relative $C'_2{}^{hu}$ concentration in the reaction mixture; the reciprocal of the serum dilution which produces one $SAC'la^{gp}$, $4^{gp},2^{hu}$ site per cell is arbitrarily defined as the number of C'^{hu} units per ml of serum (13). The titration is linear even when $C'_2{}^{hu}$ titers are as low as 1% of normal or as high as 200% of normal(13).

Results. First set rejection. Six Fisher rats received bilateral skin homografts. The dressings were removed on the sixth day at which time portions of the ghost graft could be removed from the underlying tissue which appeared moist and showed punctate areas of hemorrhage. These signs advanced until by day 10 rejection was complete. As controls, 6 Fisher rats received bilateral isologous skin grafts; such grafts showed none of the above signs of rejection at any time. Serial blood samples were taken from both groups on days 0, 4, 8, 12 and 16.

The mean preoperative serum complement level of the 5 controls and the 6 experimental animals was 40 CH_{50} with a range from 33 to 46 (Fig. 1a, 1b). The mean rise during the first 4 postoperative days was 6.2 units in the experimental group and 4.8 units in the controls, but such a rise was not apparent in all recipients. During the periods of early rejection and complete rejection the levels fell back toward the preoperative titer.

Second set rejection. Six rats were sensitized by a unilateral skin homograft. One received a second graft on the fifth day, one on the ninth day, and the remaining 4 were given second grafts on the 21st day. The second set grafts were inspected on the fourth day when signs of advanced rejection were evident. Rejection was virtually complete by day 6. Three control rats received second set isologous grafts applied on the 21st day. Blood samples were obtained from both groups on days 0, 2, 4, 6, and 8.

Compared to the 3 control isologous grafts, there is no significant variation in serum complement titer in the animals undergoing a second set rejection (Fig. 2a, 2b). The wide scatter in the serum complement titer on day 0 among the experimental animals (Fig. 2a), is presumably due to the 2 animals receiving their second grafts only 5 and 9 days after the first. The titer of these animals was still elevated due to the inflammatory response associated with the first graft.

White graft. Fisher rats demonstrated a white graft rejection when the second set homograft was applied 6 days after application of the first. A white graft was recognized as such by its failure to undergo homograft rejection of the 2nd set type. Instead, the graft gradually became dry and thickened without at any time displaying evidence of vascularization or the areas of thrombosis and hemorrhage seen in a graft undergoing classical rejection in these rats. The bed of a white graft did not bleed following stripping of the graft.

Three animals were studied during white graft rejection (Fig. 3a), and as controls 2 rats received second set homografts 14 days after the first graft and 2 rats received second set isologous grafts after the same time interval. In this experiment blood samples were taken at 0, 4, 8, 12, and 24 hours to be certain that the aforementioned studies had not missed an immediate but transient reduc-

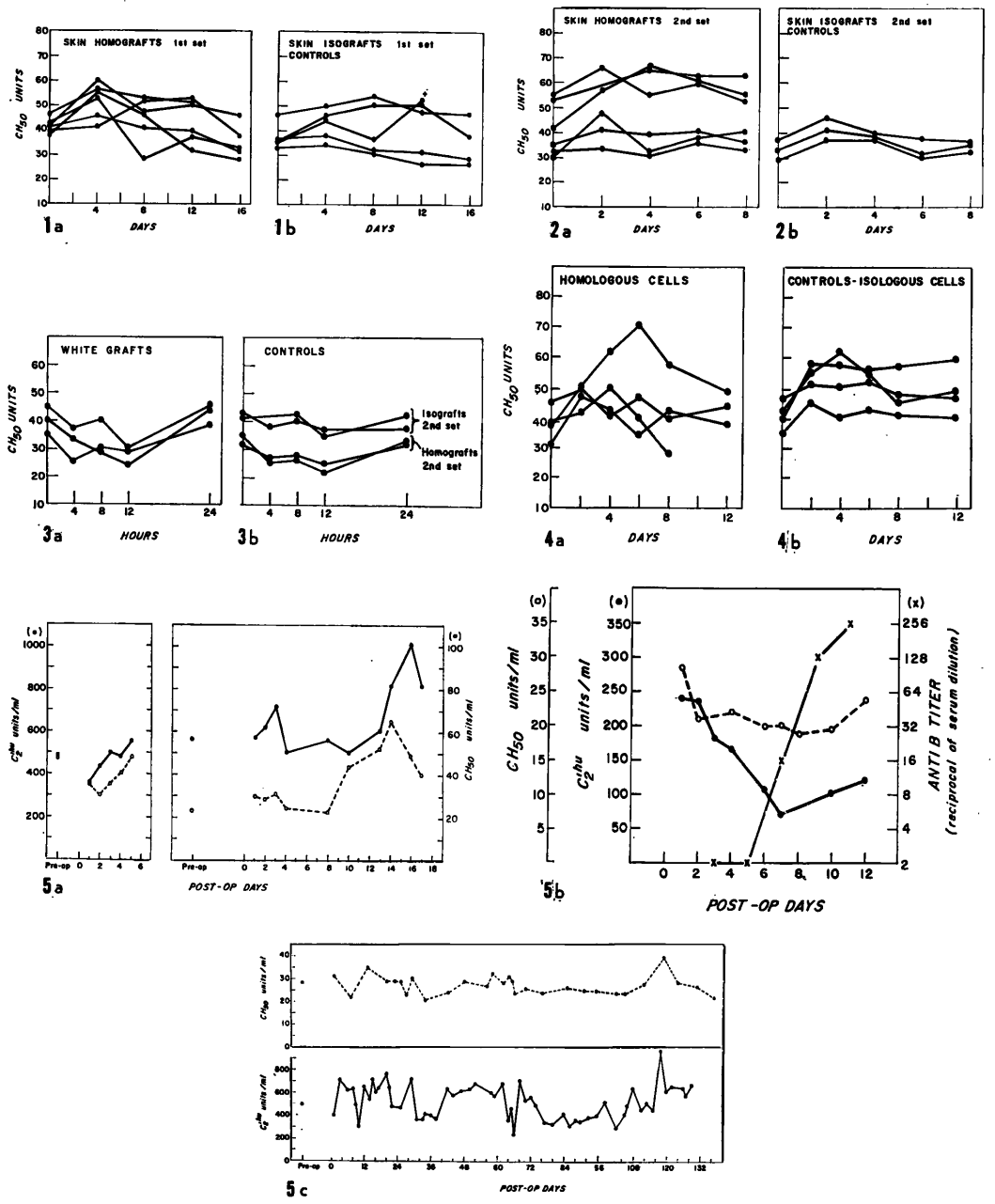

FIG. 1a, 1b. Total serum complement levels of 6 experimental (1a) and 5 control (1b) animals, following first set skin grafting. (+ animal which succumbed to pneumonia.)

FIG. 2a, 2b. Serial serum complement levels of 6 experimental (2a) and 3 control (2b) animals following second set skin grafting.

FIG. 3a, 3b. Serial serum complement levels of 3 animals which produced white grafts (3a) and 4 control animals (3b).

FIG. 4a, 4b. Total serum complement levels in 4 experimental (4a) and 4 control (4b) animals receiving injections of a cell suspension.

FIG. 5a, 5b, 5c. Total serum complement and $C'_2{}^{hu}$ levels in: (5a) Two patients who died from staphylococcal sepsis. (5b) An O negative host, who received a kidney from a B positive donor. The kidney was rejected. Anti-B antibody titer is also shown. (5c) A case of renal homotransplantation. See text for detailed interpretation.

tion in titer. Because of the frequency of sampling the animals were carefully selected for comparable weight, and the amount of blood removed on each occasion was constant.

The effect of the white graft on complement titer was indistinguishable from the control study (Fig. 3a, b).

Injection of a homologous cell suspension. Four rats received an injection of 0.17×10^9 homologous spleen and lymph node cells in Mixture 199 (Microbiological Associates, Bethesda, Md). The cells were administered subcutaneously in divided doses bilaterally in the axillary and inguinal regions. As controls, 4 animals received the same dose of isologous spleen and node cells.

Despite the minimal degree of trauma involved, the serum complement titer rose between days 0 and 2 in both groups (Fig. 4a, 4b). The initial rise and the irregular subsequent titers, especially in the experimental group, may be due to a complicated response on the part of the recipient to ectopic foreign tissue. It is not possible in this experiment to assess the degree of the specific immunological component of this response.

Human renal homografts. Of the 4 patients studied, 2 died of overwhelming staphylococcal sepsis in association with a drug-induced leukopenia. Renal function continued at near normal levels throughout and homograft rejection was not clinically evident. The striking rise in whole serum complement and $C'_2{}^{hu}$ titer in these patients preceded, and was in relation to, the fatal infection (Fig. 5a).

In the third patient there was a major blood group incompatibility, the donor being B positive and the recipient O negative. The renal homograft functioned well for 48 hours, then the urine volume declined abruptly and at 96 hours a biopsy revealed intense cellular infiltration. Rejection progressed despite immunosuppressive therapy, and by the 7th day after transplantation the homograft ceased functioning. During this 7-day period the serum complement titer fell from 28 to 20 units (Fig. 5b) and the $C'_2{}^{hu}$ titer from 240 to 72 units. On day 7 the anti-B titer began to rise, presumably because the antigenic sites on the homograft had become saturated. With the rise in antibody titer, reflecting antibody excess, the complement titer also rose.

During the 136-day period under review, the fourth patient (Fig. 5c) experienced 2 episodes of threatened rejection, days 16 to 36, and 56 to 66. On these 2 occasions there was a marked increase in number of mononuclear cells in the urine such that casts were often formed, and there was a significant diminution in the creatinine clearance which had achieved normal levels. On each occasion rejection was presumably averted by intensification of immunosuppressive therapy, Imuran, or steroids or both. On day 10 the number of mononuclear cells in the urine also increased, but this was transient and therapy was not altered. CH_{50} titers tend to be lowest during periods of threatened rejection but this pattern is no more than suggestive. $C'_2{}^{hu}$ titers show a more significant reduction in association with periods of clinical rejection. For example, the titer fell to 225 units during the second and last episode of incipient rejection but gradually climbed to pre-operative levels, 500-600 units, as renal function stabilized and the patient left the hospital. The tendency of $C'_2{}^{hu}$ and CH_{50} titers to be reduced when the urine sediment is mononuclear may be contrasted with the increase in titers on day 118 when the sediment contained almost exclusively polymorphonuclear cells prior to treatment of an acute urinary tract infection.

Discussion. In the rat, active homograft rejection, whether primary, second set, or "white," was not associated with a fall in serum complement titer. Titers were measured throughout the period of rejection (Fig. 1, 2) in the case of the first and second set, and serially during the first 24 hours after grafting for the white graft (Fig. 3). Even when homologous cells were delivered in dissociated form by injection to minimize surgical trauma, no fall in serum complement was observed (Fig. 4). The failure to demonstrate a fall in titer in association with skin graft rejection may be due in part to masking by a rise in titer secondary to the inflammation aroused by the foreign tissue or cells.

The data in 2 of the patients suggest that under some circumstances rejection can be associated with a moderate reduction in CH_{50} titer (Fig. 5b, 5c). This is emphasized by contrasting the pronounced rise in titer which occurs with obvious sepsis (Fig. 5a, 5c). However, in terms of further investigation, the pattern obtained with the $C'_2{}^{hu}$ assay is much more promising than that observed with the CH_{50} determination.

The whole complement titer involves at least 7 components(16), but with certain qualifications depends mostly on the supply of second component and third component factors(2). Antigen-antibody aggregates reacting with serum complement so as to achieve the 1,4,2 state (AgAbC'1,4,2) deplete the serum of the second component by a cyclic process involving decay back to the 1,4 state and utilization of additional C'_2 to restore the 1,4,2 complex(17,18). In this manner a reduction in C'_2 titer can occur irrespective of the participation of C'_3 factors, and without being fully reflected in the whole complement (CH_{50}) determination. This is illustrated by the findings during the homograft rejection in patient 3 (Fig. 5b); the $C'_2{}^{hu}$ titer fell 70% from 240 to 72 units, while the CH_{50} titer was only reduced 29%. In view of the blood group incompatibility this complement utilization can be attributed to the interaction of the anti-B isohemagglutinin with the specific sessile antigen known to be present in renal tissue(19). The relevance of this complement fixation to the rejection process is unknown. In patient 4 (Fig. 5c) there was no major blood group incompatibility, but the $C'_2{}^{hu}$ titer was reduced approximately 50% in association with periods of threatened rejection and returned to pre-operative levels as the patient's renal function improved. The interpretation of these $C'_2{}^{hu}$ findings in man and their possible value in the diagnosis of threatened rejection requires further clinical study. A specific titration for rat C'_2 is not yet available. However, even component titrations may have important limitations in a situation as complex as homograft rejection in that immunologic complement fixation may be masked by their overproduction or release associated with inflammation. Complement component turnover studies may be the only way to measure utilization in complex *in vivo* reactions.

Summary. In the rat, active homograft rejection of skin, whether first set, second set, or "white" does not alter the serum whole complement titer (CH_{50}). During renal homograft rejection in man there is a small reduction in the CH_{50} titer, and a pronounced diminution in the titer of the second component of human complement ($C'_2{}^{hu}$), presumably reflecting *in vivo* complement fixation.

1. Mayer, M. M., *Progr. Allergy,* 1958, v5, 215.
2. ———, *Cancer Res.,* 1961, 1262.
3. Muschel, L. H., Treffers, H. P., *J. Immunol.,* 1956, v76, 11.
4. Goldberg, B., Green, H., *J. Exp. Med.,* 1959, v109, 505.
5. Green, H., Fleischer, R. A., Barrow, P., Goldberg, B., *ibid.,* 1959, v109, 511.
6. Bloch, K., Kourilsky, F. M., Ovary, Z., Benacerraf, B., *ibid.,* 1963, v117, 965.
7. Simonsen, M., *Acta Path. et Microbiol. Scand.,* 1953, v32, 36.
8. Favour, C. B., Murray, J. E., Wemyss, C. T., Colodny, A., Miller, B. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 352.
9. Hume, D. M., Merrill, J., Miller, B. F., Thorn, G. W., *J. Clin. Invest.,* 1955, v36, 327.
10. Boltax, A. J., Fischel, E. E., *Am. J. Med.,* 1956, v20, 418.
11. Fife, E. H., Hook, W. A., Muschel, L. H., PROC. SOC. EXP. BIOL. AND MED., 1962, v110, 526.
12. Russell, P. S., *Transplantation,* Ciba Foundation Symposium, 1962, 350.
13. Austen, K. F., Beer, F., *J. Immunol.,* 1964, in press.
14. Billingham, R. E., Medawar, P. B., *J. Exp. Biol.,* 1951, v28, 385.
15. Kabat, E. A., Mayer, M. M., *Exp. Immunochemistry,* 1961, 2nd ed., 135.
16. Rapp, H. J., Borsos, T., *Science,* 1963, v141, 738.
17. Mayer, M. M., Levine, L., Rapp, H. J., Marucci, A. A., *J. Immunol.,* 1954, v73, 443.
18. Borsos, T., Rapp, H. J., Mayer, M. M., *ibid.,* 1961, v87, 326.
19. Szulman, A., *J. Exp. Med.,* 1962, v115, 977.

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