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Complement in Graft Versus Host Disease: I. Depletion of Complement Components during a Systemic Graft Versus Host Reaction in the Rat[1] (38499)

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Introduction. In patients who have received a bone marrow transplant for treatment of immunodeficiency disease (1), aplastic anemia or leukemia (2–4), graft versus host (GVH) disease remains a major problem. The major mechanism of the GVH reaction is thought to be a cell-mediated immune response on the part of the thymus-derived or T-cell population of the donor against the immunologically incompetent host. Clinical manifestations of the GVH disease are well described (5). Initial lymphoid hypertrophy followed by lymphoid atrophy and the increased susceptibility to infection are major characteristics, the latter often contributing to the death of the host. However, the pathogenesis of the GVH disease is not well understood.

Recently, more attention has been directed towards analysis of, and contributions made by the host in the development and expression of the GVH reaction. The majority of the proliferating cells in the enlarged spleen and lymph nodes are of host origin (6, 7). Elkins (8, 9) has emphasized the importance of the host leukocyte in the development of the local GVH reaction under the renal capsule. Lafferty (10) has emphasized the importance of host hematopoietic cells in the pathogenesis of the graft versus host reaction in both birds and mammals. Other studies (11) employing a local GVH reaction have demonstrated the importance of host marrow-derived cell constituents. Since by the very nature of GVH models the host is not able to mount a specific immune response against donor cells, these studies suggest a nonspecific recruitment and proliferation of host cells after the initiation of the GVH reaction by donor lymphocytes. This nonspecific immunologic response could represent the activation of various inflammatory effector mechanisms. One such effector mechanism of course, is the complement system whose biologically active fragments are important in cell recruitment (12) and host defense against infection (13).

In the experiments described in this paper, we have examined the role of complement (C) in GVH disease. F_1 hybrid rats were given a systemic GVH disease by the injection of spleen lymphocytes from parent strains, and the serum complement was determined. These studies demonstrated a depletion of serum complement, especially of the earlier C components C2 and C4, during systemic GVH disease. The possible mechanisms for the depletion of these complement components are discussed.

Material and Methods. Systemic GVH reaction. F_1 hybrid rats [Lewis × Brown Norway (L–BN)] or [Fischer 344 × Brown Norway (F–BN)] approximately 4–5 weeks old weighing 60–80 g and the inbred parental adult rats, Lewis (Le) or Fischer 344 (F) weighing 175–200 g were employed in these studies (Microbiological Associates, Bethesda, MD). Spleen cells from the parental adult rats were prepared either as homogenates or by Ficoll–Hypaque separation as described previously (14), and were injected intravenously into a tail vein of the respective F_1 hybrids. Rats of control groups were injected with the spleen cells of F_1 hybrid rats. A systemic GVH disease was obtained with 200×10^6–400×10^6 spleen cells.

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Rat serum. Blood was obtained by cardiac puncture (25 g needle on tuberculin syringe) under ether anesthesia from the F_1 hybrid rats prior to and at various intervals after the induction of the systemic GVH. The blood was allowed to clot at room temperature and kept overnight at 4°. The serum was then separated and stored at −70°.

Buffer for complement assays and intermediate cells. Veronal-buffered saline, pH 7.4 containing 0.1% gelatin and 0.01 *M* disodium ethylene-diamine tetracetic acid (EDTA); and dextrose veronal buffered saline containing 0.1% gelatin, 0.00015 *M* calcium and 0.0005 *M* magnesium ($GGVB^{++}$) were prepared as previously described for the measurement of C components (15). Sensitized sheep erythrocytes (15) and the cellular intermediates EAC1, EAC 4_{hu}, and $EAC1_{gp}4_{hu}$ were prepared according to the method of Borsos and Rapp (16). Functionally pure C components for the assay of C3, C5 and C6 and the $EAC1_{gp}4\text{–}7_{hu}$ for use in the assay of C8 were obtained from Cordis Laboratories (Miami, FL). Partially purified C2 was prepared from guinea pig serum (17).

Measurement of rat C components. The early C components C1, C4 and C2 were measured hemolytically as previously described (15). Rat serum at a dilution of 1:25 in 0.04 *M* EDTA-GVB was used as a source of C3–C9 components (16). The 50 percent endpoint was calculated by interpolation on semilogarithm paper.

The later C components C3, C5, C6 and C8 were assayed according to the methods of Nelson *et al.* (17) as modified by Geiger *et al.* (19) and Miyakana *et al.* (18).

Because of the variability of the cellular intermediates, all serum samples obtained prior to and after the induction of the GVH disease from one individual animal were assayed together on the same day for each C component. Utilizing the CH50 units, a ratio to day 0 (prior to GVH induction) was calculated and expressed as the percentage change from baseline. The experimental error of a titer on any single day was less than 10%.

Results. In the first series of experiments, L–BN F_1 hybrid rats were injected intravenously with a suspension of 200×10^6 or 400×10^6 Le spleen cells, while control F_1 hybrid rats were given a suspension of equal numbers of F_1 spleen cells. Clinical GVH disease became evident approximately 5–7 days after spleen cell injection and was manifested by a dermatitis, loose stools, and then infection and sometimes death. Body weights were variable in that some animals lost weight, but others gained weight secondary to the accumulation of ascitic fluids.

Of three rats receiving 200×10^6 Le spleen cells, all animals developed deficiencies of C2 (20–76% below baseline) and C4 (75–90% below baseline) between days 4 and 7 after injection of the spleen cells. This period just antedates or coincides with the clinical manifestations of GVH disease. C1 remained unchanged with the exception of one rat in this group which showed a deficiency of this C component (40% below baseline) while a reduction in C5 was seen in another rat (40% below baseline). In the group of three rats receiving 400×10^6 Le spleen cells, serum complement levels of the rats showed a decrease, primarily C2 (50–76%) and C4 (90–98%). A marked increase in C components concomitant with the onset of infection was seen in most animals.

In the control group (six rats) receiving L–BN F_1 hybrid spleen cells, no clinical signs of GVH disease developed. In general, a small reduction (20–30%) of C components occurred on day 4. After this period a marked increase in C components was evident. Only one rat in the control groups developed a significant fall in C components. The reason for this is not clear.

Two important points were brought out by further experiments with GVH reaction. First, the recipient F_1 hybrid rats had to be of the correct size and age range (60–80 g, 4–5 weeks) to obtain GVH disease. In two series of experiments in which the F_1 hybrid rats did not manifest clinical GVH disease, a reduction in C components did not occur. A second factor is the introduction of an inflammatory process with the initiation of the GVH disease, which will produce a marked increase in C components as often seen in acute inflammatory states (20, 21).

In the previous series of experiments, C was not measured until day 4 after initiation of the GVH reaction. It was possible that the injection of such large numbers of spleen cells had a nonspecific anticomplementary effect causing a fall in C components which persisted until the first serum sample was obtained on day 4. To study this possibility, serum was obtained earlier, i.e. on day 2. Since the spleen cell suspension contained some red cells and reticulum components, lymphocytes were semi-purified by Ficoll-Hypaque separation for initiation of the GVH reaction. Fischer-Brown Norway F_1 hybrid rats were injected intravenously either with 400 $\times$ 10^6 F spleen cells, or 200 $\times$ 10^6 F lymphocytes separated by Ficoll-Hypaque gradient. The control groups were injected with either 400 $\times$ 10^6 F-BN F_1 spleen cells or 200 $\times$ 10^6 F-BN F_1 lymphocytes from Ficoll-Hypaque separation. Each group consisted of 4 animals except for one experimental group (three animals) given 200 $\times$ 10^6 F lymphocytes.

Unlike the previously described experiments, clinical GVH disease was seen on day 10 instead of day 4 in both experimental groups, a difference attributable to the differences in the rat strain used. The first sign was erythema of the skin, followed by a dermatitis, loose stools, secondary infection, weight loss, and in two animals, ascites. None of the control groups of animals became sick. Three of the 4 animals injected with 400 $\times$ 10^6 F spleen cell suspension had died by day 20, while only one of the three rats given 200 $\times$ 10^6 F lymphocytes died.

As shown in Fig. 1, C1 and the total serum protein exhibited no differences in any of the rats studied. However, as seen in the previous series of experiments, both C2 and C4 fell dramatically when compared with the control groups, on days 10 and 13 ($P < 0.05-<0.001$, Fig. 2). When the later C components were examined, a deficiency of C3, C5 and C6 was also demonstrated on days 10 and 13. The changes in these components however, was not as dramatic

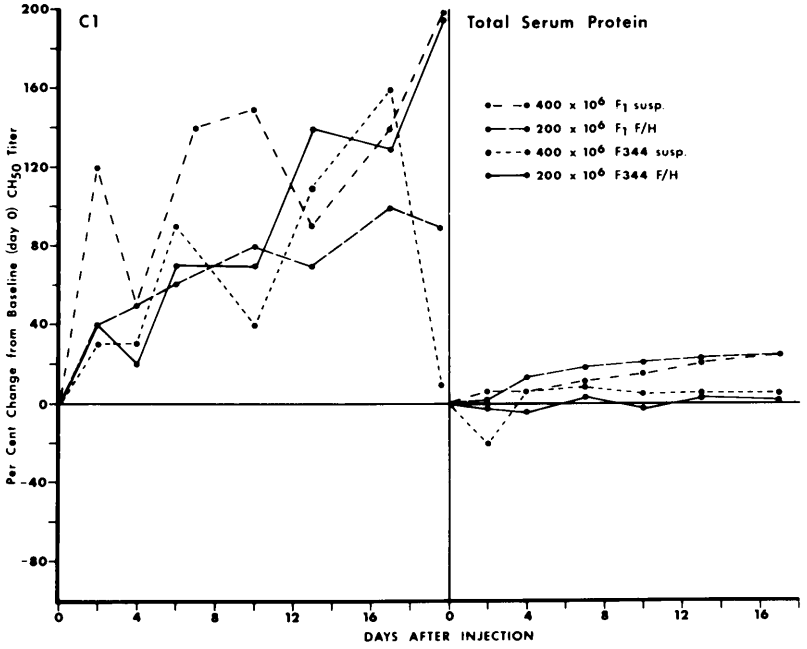

FIG. 1. Serum complement C1 and total protein during GVH reaction in F-BN F_1 hybrid rats injected on day 0 intravenously with spleen cell suspension or lymphocytes from Ficoll-Hypaque separation. Control groups received the appropriate F-BN F_1 hybrid cells. Each point represents the mean of four rats, except the experimental group given F344 F/H lymphocytes from day 0, in which there were three rats Each mean is expressed as the percent change. Total serum proteins were measured by the Folin method.

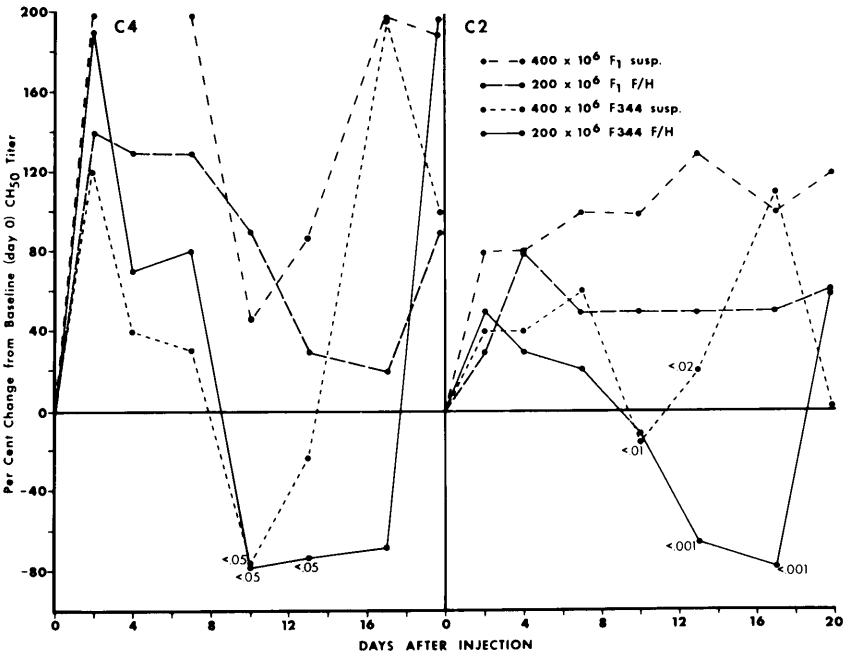

FIG. 2. Serum complement C4 and C2 during GVH reaction in F-BN F_1 hybrid rats. Statistically significant *P* values as compared to the respective control group are indicated in the figure.

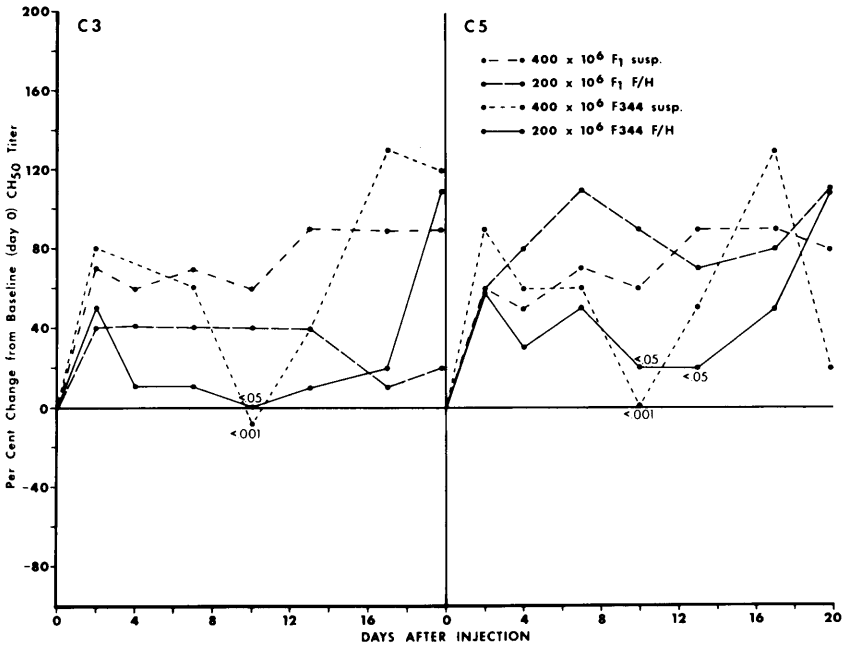

FIG. 3. Serum complement C3 and C5 during GVH reaction in F-BN F_1 hybrid rats.

as that of C2 and C4, but nonetheless statistically significant when compared to the control groups ($P < 0.05$ to <0.001, Fig. 3 and 4). Interestingly, the fall in C8 was slightly delayed until days 13 and 17. As seen previously, a marked increase in C components often occurred when the animals became clinically infected (days 17–20).

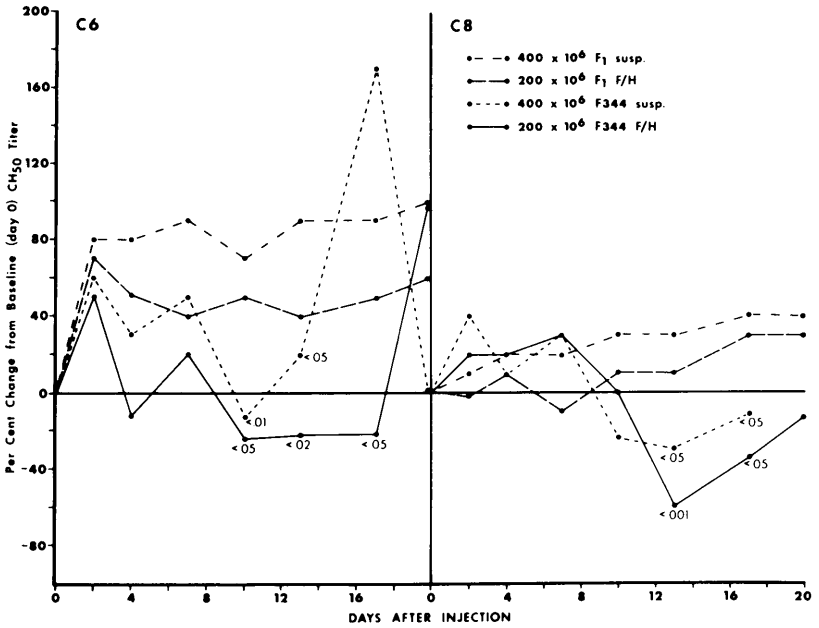

FIG. 4. Serum complement C6 and C8 during GVH reaction in F-BN F_1 hybrid rats.

Discussion. The GVH reaction was but a laboratory model in experimental animals until recently when marrow transplantation in humans became a reality (22). Since GVH disease is often a fatal illness, it becomes important to understand all the facets of this disease complex. It is quite clear that the initiating forces in the GVH reactions are dependent on the thymus-derived lymphocyte (23) of the donor; however, examination of the host as playing a major part in the mechanism(s) by which GVH is perpetuated has recently come under scrutiny (9–11). Thus, it seems logical to examine the involvement of C as a major host defense system in GVH disease.

Studies of the C system in GVH reactions have received little attention. Fife *et al.* (24) observed a decrease in whole hemolytic C in newborn rats undergoing runt disease. Later Gewurz *et al.* (25) could not demonstrate complement participation in the chick embryo GVH reaction by studies of whole complement. However, these same investigators described a patient with immunodeficiency disease whose early C components fell markedly during GVH disease following a marrow transplant (26). More recently we also observed a depletion of both early and late C components in a patient with severe combined immunodeficiency disease during a GVH reaction initiated by a marrow transplant from her father (27).

In the present study we have demonstrated the depletion of C components during systemic GVH disease in the rat. The deficiency of C seems to involve primarily the early components C4 and C2. The serum concentration of any C component in time reflects a balance between synthesis and utilization. Thus, the depletion of C components in our study could of course reflect immunological activation, impaired synthesis or increased catabolism. Kinetic studies are necessary to answer these questions. The unchanged levels of C1 probably is related to increased concentrations of C1 in the rat or increased synthesis. However, the depletion of C4 and C2 together with the fall in C3, C5, C6 and C8 suggest a sequential activation of C through the classical pathway.

Infection and overwhelming sepsis remain one of the major life threatening manifestations of GVH disease. In our rat study we observed a fall in the C components at the very beginning of clinical GVH disease. Infection, sepsis and occasionally death followed later. The depletion of C, therefore, could have led to an increased sus-

ceptibility to infection by comprising one of the host's most important effector systems of inflammation.

The mechanism of C depletion in our studies is not clear. If the deficiency of C components represents immunologic activation, two possible mechanisms may exist. In both series of systemic GVH experiments, the depletion of C components appeared just prior to, or with the initial clinical GVH disease. As Levine and others (28–30) have shown, considerable proliferation of mononuclear blast-like cells occurs as an initial process in the GVH reaction. These proliferating blast-like cells responsible for the splenomegaly and lymphoadenopathy may activate the C system via the early C components as seen in our study. Furthermore, Ferrone *et al.* (31) described a "nonspecific" activation of the early C components by cultured WI-L2 lymphoid cells while normal peripheral lymphocytes did not activate the C sequence. Thus, the membrane characteristics of the cells involved in the GVH reaction may undergo changes during blast transformation which lead to the activation of serum C.

Finally, although the major immune response during the GVH reaction may be mediated by T-cells, the participation of the humoral immune system or B-cells has been recognized (32). A Coombs positive hemolytic anemia is occasionally seen during GVH disease (33, 34). Streilein and Stone (35) demonstrated multiple types of autoantibodies in hamsters with GVH disease. It is possible that in our studies of the systemic GVH reaction in the rat, circulating autoantibodies or immune complexes may have activated the C sequence. Against this view, however, is the observation that depletion of C components occurred prior to, or at the beginning of clinical manifestations of the GVH disease.

Further studies certainly will be necessary to elucidate and clarify all of the relationships that are or may be involved in these interactions, but the present observations seem to us to establish an important link between GVH and significant perturbations of the C system in the rat.

Summary. Serum complement (C) and C components were examined during a systemic graft versus host (GVH) reaction in the rat. In our series of experiments (Lewis × Brown Norway) F_1 hybrid rats (60–80g) were given 200×10^6 or 400×10^6 Lewis spleen cells intravenously. Clinical GVH disease appeared 5–7 days after cell injection. Five of six rats in the experimental groups had a fall in levels of serum C2 (20–76%) and C4 (75–98%). Only one of six rats in the control group had a significant fall in C components.

In a subsequent experiment (Fischer 344 × Brown Norway) F_1 hybrid rats (60g) were given 400×10^6 Fischer 344 spleen cells or 200×10^6 Fischer 344 Ficoll–Hypaque separated spleen lymphocytes. Clinical GVH disease in this instance appeared on day 10. As in the previous experiments C2 and C4 fell markedly, 20–60% and 60–80%, respectively, from baseline titers. The control groups did not have a significant fall in C2 or C4. Further examination showed reduction in C3, C5, C6 and C8 suggesting a sequential activation of the C system via the classical pathway.

We have postulated that the cells undergoing blast transformation may be activating the C system through membrane changes during the GVH reaction. Furthermore, the deficiency of C and C components during GVH disease may contribute to the increased susceptibility of the host to infection and sepsis.

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