Cytogenetic Engineering *In Vivo*: Restoration of Biologic Complement Activity to C5-Deficient Mice by Intravenous Inoculation of Hybrid Cells

(mouse splenic macrophages/chicken erythrocytes/mouse kidney cells/genetic repair/MuB1 protein)

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ABSTRACT Splenic macrophages were identified as at least one source of C5 elaboration in normal mice. Hybrid cells were formed from splenic macrophages from C5-deficient mice and either kidney cells from mice with normal amounts of C5 or chicken erythrocytes. These hybrids elaborated C5 *in vitro*. C5-Deficient mice inoculated with these hybrid cells developed, in their serum, antigenically active mouse C5, as well as both hemolytic and biologic complement activity. These studies demonstrate the feasibility of genetic "repair" in mammals.

Lack of hemolytic and biologic complement activity in the serum of B10.D2/o mice is the consequence of an inherited deficiency in the synthesis of MuB1 protein, which is the mouse analogue of the fifth component (C5) of the complement system (1). Production of MuB1 is controlled by a single dominant gene (2). Deficient mice produce no protein with MuB1 activity detectable either by functional (3) or immunochemical (3) assays and, indeed, produce antibody against MuB1 when injected with normal mouse serum. Furthermore, the deficient strain is closely related (previously thought to be coisogenic) to another strain, B10.D2/n, which produces MuB1 and has an intact complement system.

In a previous study (4), we reported *in vivo* restoration of hemolytic activity to the serum of B10.D2/o mice by intravenous inoculation either of B10.D2/n spleen cells or of hybrid cells formed from B10.D2/o spleen and B10.D2/n kidney cells or B10.D2/o spleen cells and chicken erythrocytes. We now report a restoration to deficient animals of not only hemolytic, but biologic complement activity as well. We, moreover, show that it is MuB1 protein which is restored and present information on the genetic control of the MuB1 locus in mice. These studies demonstrate the feasibility of genetic "repair" experiments in mammals and form a prototype for further investigation.

MATERIALS AND METHODS

Animals. Adult male B10.D2/o and B10.D2/n mice were purchased from the Jackson Laboratory, Bar Harbor, Me. Serum from each mouse used was assayed for hemolytic complement activity. Titers in B10.D2/n mice ranged from

8 to 35 CH₅₀ units per ml. None of the B10.D2/o mice used in our experiments had detectable serum hemolytic complement activity. Skin grafts exchanged between B10.D2/n and B10.D2/o mice survived more than 150 days. Male BALB/c mice were obtained from the Animal Production Branch of the National Institutes of Health. Adult White Leghorn roosters were obtained either from the National Naval Medical Center, Bethesda, Md., or from Dr. Joseph W. Beard, Duke University.

Cell Preparation. Mouse or chicken spleen cells were teased from the splenic capsule and passed through a fine mesh stainless steel screen. Adherent and nonadherent cell populations were obtained by incubating 10⁸ spleen cells in a 75-cm² plastic culture flask (Falcon Plastics, Inc.) for 45 min at 37°. Nonadherent cells were removed by washing with modified (5) Hank's balanced salt solution and were incubated in a second flask. The resultant nonadherent cells were gently decanted, and erythrocytes were lysed with Tris-buffered hypotonic NH₄Cl (6). At least 95% of the adherent cells ingested carbon particles, while 90% of the cells nonadherent after two incubations appeared morphologically as small lymphocytes and failed to phagocytose carbon particles. Preparations of kidney, liver, and brain cells were derived by trypsinization of washed, minced fresh tissue and were used in the primary cultures. Mouse-skin fibroblasts were obtained by trypsinization of explant cultures. Cells were washed three times in cold Hank's solution. Fresh adult chicken erythrocytes were washed four times at room temperature in dextrose-gelatin-Veronal buffer and suspended in ice-cold Hank's solution. With each wash the buffy coat was discarded, and the final cell suspension contained no leukocytes.

Preparation of Hybrid Cells. Experimental Design. Allantoic fluid infected by Sendai virus was prepared as described (7) and inactivated, just before use, with 0.06% β -propiolactone (8). This inactivated allantoic fluid had no residual infectivity for embryonated eggs.

Table 1 presents the experimental groups and the number of each cell type used. Cells were hybridized by mixing, in a siliconized tube, the cell suspensions in an equal volume of allantoic fluid containing inactivated Sendai virus and incubating the mixture for 30 min in ice, followed by 60 min at 37°. Unhybridized controls received uninfected allantoic fluid treated with β -propiolactone instead of allantoic fluid infected with Sendai virus. Cells were sedimented at 200 $\times$ g

Abbreviations: C5, fifth component of the hemolytic complement system; CH₅₀, amount of complement that lyses 50% of a standard number of sensitized erythrocytes.

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TABLE 1. *Experimental grouping*

Group	B10.D2/n kidney cells	B10.D2/n splenic macrophages	Adult chicken erythrocytes	B10.D2/o kidney cells	B10.D2/o splenic macrophages	Inactivated SAF
1	1×10^8 * (1 ml)				1×10^8 (1 ml)	+
2	1×10^8 (1 ml)				1×10^8 (1 ml)	—
3	2×10^8 (2 ml)					+
4					2×10^8 (2 ml)	+
5				1×10^8 (1 ml)	1×10^8 (1 ml)	+
6		2×10^8 (2 ml)				+
7		2×10^8 (2 ml)				—
8			1×10^{10} (1 ml)		1×10^8 (1 ml)	+
9			1×10^{10} (1 ml)		1×10^8 (1 ml)	—
10			1×10^{10} (1 ml)			+
11	1×10^8 (1 ml)		1×10^{10} (1 ml)			+

* Total cells added to each reaction mixture. SAF, allantoic fluid infected with Sendai virus.

for 10 min and washed three times with medium 199 containing 100 units per ml of penicillin and 100 μ g per ml of streptomycin but no serum. At least 75% of the cells in groups 1, 3, 4, 5, and 6 (Table 1) contained two or more nuclei. More than 95% of the mouse cells in groups 8 and 11 contained at least one chicken erythrocyte nucleus.

For experiments on the *in vitro* production of C5 by hybrid cells, the cells were resuspended in 6 ml of culture medium consisting of Eagle's minimal essential medium supplemented with Eagle's nonessential amino acids, 0.5% ovalbumin, 50 μ g/ml of insulin, and 0.1 μ g/ml of hydrocortisone; 1.5 ml was placed in each of four roller tubes. The tubes were incubated at 37° on a roller apparatus, and at 0, 6, 24, 48, 72, and 96 hr after initiation of the cultures, 0.1-ml cell-free aliquots were removed. Half of each sample was added immediately to 0.05 ml of fresh C5-deficient serum from B10-D2/o mice; the other half was added to 0.05 ml of heat-inactivated (56° for 45 min) B10.D2/o serum, and the mixtures were assayed for hemolytic complement activity.

FIG. 1. Cellular site of C5 synthesis. Mouse cells from various organs were cultured at 5×10^6 cells per culture in serum-free medium. Aliquots of the culture supernatants were removed at intervals and added to an equal volume of C5-deficient mouse serum. The hemolytic activity of the mixture was assayed on sensitized sheep erythrocytes. Spleen cells, unfractionated, ●; adherent spleen cells (from 5×10^6 spleen cells), ■; nonadherent spleen cells, ▲; kidney cells, ▼; brain cells, ●; skin fibroblasts, ◆; liver cells, —. Open circles represent the hemolytic activity of spleen-cell supernatants added to heated C5-deficient mouse serum. Curves show the average of four replicate cultures with standard errors.

For *in vivo* experiments, the cells were resuspended in 1 ml of medium 199 and 0.25 ml was inoculated intravenously into each of four B10.D2/o mice per group. Mice were bled on days 0, 3, 6, 10, 14, 21, and 30 after inoculation, and the sera were stored at -80°. All mice survived the entire experiment.

Mouse Complement Titration. Mouse complement was titrated by the method of Terry *et al.* (9), using 2.5×10^8 maximally-sensitized sheep erythrocytes per ml. The concentration of unhemolyzed cells was determined as described (3).

Quantitation of Mouse-Serum Neutrophil Chemotactic Activity. Mouse serum (0.05 ml) was incubated at 37° for 60 min either with gelatin-Veronal buffer containing 200 μ g/ml of *Salmonella typhosa* endotoxin or with gelatin-Veronal buffer alone. Sera so treated were assayed for chemotactic activity with BALB/c peripheral blood neutrophils (10) by a modification (11) of the method described by Boyden (12).

Identification of MuB1 by Immunodiffusion. Myeloma proteins of BALB/c origin prepared by ion-exchange methods

FIG. 2. *In vitro* elaboration of C5 by hybrid cells. Cells from group 1 (■), group 2 (▲), group 3 (●), group 4 (▼), group 8 (●), and group 9 (—) (see Table 1), were cultured in serum-free medium, and aliquots were removed at intervals and assayed for ability to restore C5 hemolytic activity as in Fig. 1. Open squares and circles were from mixtures of heat-inactivated C5-deficient serum and supernatants from group 1 and group 8, respectively.

Fig. 3. Development of hemolytic complement activity in the serum of B10.D2/o mice injected with B10.D2/o spleen:B10.D2/n kidney hybrid cells (group 1). Each of the curves shows the results from an individual animal. Complement activity is expressed as percentage of lysis of sensitized sheep erythrocytes. Sera from control groups 2, 3, 4, and 5 had no hemolytic activity.

Fig. 4. Development of hemolytic complement activity in the serum of B10.D2/o mice injected with B10.D2/o spleen:chicken erythrocyte hybrid cells (group 8). Each of the curves shows the results from an individual animal. Complement activity is expressed as percentage of lysis of sensitized sheep erythrocytes. Sera from control groups 9, 10, and 11 had no hemolytic activity.

are occasionally associated with the fifth component of complement (MuB1). Such a myeloma protein, T-15, of the IgA class was used to raise an antiserum in A/He mice that identified two different specificities, MuB1 and a T-15 idiotype. The antibody to the T-15 idiotype reacted with only that immunogen and has no relevance to this study. The antibody directed to the MuB1 specificity was easily distinguished and was identified by the pattern of precipitation with sera from 50 different inbred strains and agreed completely with that found with a known antiserum against MuB1 (RF anti-RIII). The antiserum against MuB1 used in this study, in contrast to the usual antiserum against MuB1, was species specific and did not precipitate with human, rabbit, calf, guinea pig, chicken, or goat serum. Mouse sera to be assayed for MuB1 activity were reacted with this antiserum against MuB1 in double-diffusion agar plates.

RESULTS

Cellular Site of C5 Synthesis. Before any gene complementation studies could be attempted, it was necessary to know what cells normally elaborate C5. Cultures, each containing 5×10^6 cells from a different organ of B10.D2/n mice (with normal C5), were initiated in serum-free culture medium. Cultures were incubated at 37° on a roller apparatus, and, at intervals, supernatants were examined for their ability to restore hemolytic activity to C5-deficient serum. By trypan blue exclusion, all cultures contained at least 80% viable cells on day 4. Fig. 1 shows that only spleen-cell supernatants restored hemolytic activity to C5-deficient serum. Since spleen-cell supernatants did not render heated C5-deficient serum hemolytic, we can conclude that the spleen cells did not elaborate a nonspecific hemolysin. Within the spleen-cell population, only those cells able to adhere to plastic and ingest carbon particles secreted C5. These cells morphologically appeared as macrophages. Cultures containing as many as 10^8 kidney, brain, liver, or nonadherent spleen cells still produced no C5. An homogenate of 10^7 splenic macrophages, clarified at $20,000 \times g$ for 30 min, did contain C5 activity, while similar preparations from splenic lymphocytes or from

brain, kidney, or liver cells had no C5 activity. The kinetics of C5 appearance suggested that it was actively secreted by the cultured macrophages rather than simply released from a cell-bound state. Furthermore, addition of puromycin ($50 \mu\text{g/ml}$) to the culture medium on day 0, but not on day 3, prevented the appearance of C5-restoring activity in medium removed on day 4.

In Vitro Synthesis of C5 by Hybrid Cells. Hybrid cells were formed from B10.D2/n kidney cells and B10.D2/o splenic macrophages. Hybrids were also formed from B10.D2/o splenic macrophages and adult chicken erythrocytes. None of these individual cells contained or secreted C5. Table 1 presents the various hybrid and unhybridized cell combinations used. Cultures of the cells were established, and, at intervals, medium was withdrawn and assayed for its ability to restore hemolytic activity to C5-deficient serum. Fig. 2 shows that cultures of B10.D2/o spleen:B10.D2/n kidney (group 1) and B10.D2/o spleen:chicken erythrocyte (group 8) hybrids^{||} produced C5-restoring activity, while unhybridized mixtures of the same cell types (groups 2 and 9) did not. Hemolysis was not due either to Sendai virus alone or to interaction of the virus with any one of the cell types since supernatants from groups 3, 4, and 10 failed to restore C5 activity. Hybrid cells remained viable for at least 10 days, at which time the experiment was terminated.

In Vivo Restoration of C5 Hemolytic Activity. Figs. 3 and 4 demonstrate restoration of hemolytic complement activity to B10.D2/o mice after injection of B10.D2/o spleen:B10.D2/n kidney (group 1) and B10.D2/o spleen:chicken erythrocyte (group 8) hybrid cells, respectively. These results were very similar to those reported in our earlier study (4). In the present study, however, splenic macrophages were used rather than an unfractionated spleen-cell suspension. The activity appeared by day 3, rose to a peak between days 3 and 6, and thereafter began a slow decline until day 21, when it had become undetectable. Sera from control groups

^{||} Colon (:) denotes cell hybridization.

FIG. 5. Development of chemotaxis-generating activity in the serum of B10.D2/o mice after infusion of hybrid cells. Serum from two animals in group 1 (●) and two animals in group 8 (■) was taken on the indicated day and incubated with 200 μ g of endotoxin. The reaction mixtures were tested for neutrophil chemotactic activity (13), and the results were expressed as migrating cells per high-power field. Controls consisted of the serum from the same groups incubated in the absence of endotoxin (open symbols). Results with each animal's serum are plotted individually.

2, 3, 4, 5, 9, 10, and 11 at no time exhibited detectable hemolytic activity. Sera from animals receiving B10.D2/n spleen cells, with or without Sendai virus (groups 6 and 7), did show hemolytic activity with a peak of 55–75% lysis by day 3. This activity also disappeared by day 21. None of the samples had nonspecific hemolytic activity when tested against unsensitized sheep cells.

Restoration of Chemotaxis-Generating Activity to C5-Deficient Mice. Activation of a normal C5 molecule involves its cleavage into two polypeptide chains (14), each with disparate biological activities. The larger fragment (C5b) binds to the immune complex and there facilitates binding and activation of C6 and C7 and continuation of the hemolytic cascade. The smaller fragment (C5a) does not participate in the hemolytic system but is responsible for neutrophil chemotactic and anaphylotoxic activity in serum activated by such inflammatory agents as endotoxin (15). To assure that C5 containing both fragments was restored to deficient animals by injection of hybrid cells, aliquots of the same sera that had been titrated for hemolytic activity (Figs. 3 and 4) were examined for their ability to generate neutrophil chemotactic activity upon incubation with endotoxin. Fig. 5 shows that deficient mice developed chemotaxis-generating activity within 3 days after injection of B10.D2/o spleen:B10.D2/n kidney (group 1) or B10.D2/o spleen:chicken erythrocyte (group 8) hybrid cells. The appearance and disappearance of chemotaxis-generating activity corresponded to that of the hemolytic activity (Figs. 3 and 4). Peak chemotactic activity was about 60% of that generated by endotoxin in serum from B10.D2/n animals. No chemotactic activity could be generated in sera from animals in groups 2 and 9.

Restoration of MuB1 Antigen Activity. Figs. 3–5 show that the “restored” animals had functional C5 activity, but the possibility remained that this was not due to a restoration of MuB1 antigen. Fig. 6 presents the results of double-immunodiffusion studies with an antiserum against MuB1 (A/He anti-T15) against aliquots of the same experimental

sera previously assayed for C5 functional activity. No lines of precipitation were seen with sera drawn immediately after cell injection (time 0) in groups 1, 6, or 8. Precipitin lines appeared by day 3 after cell injection in all three groups. The intensity of the reaction with “restored” sera drawn at different intervals after cell injection correlated well with the degree of hemolytic and chemotaxis-generating activity of the sera. There was complete coalescence of precipitin lines produced with DBA/1 serum (C5 normal) and with serum from B10.D2/o mice “restored” with mouse:chicken hybrid cells (group 8). There was likewise coalescence between lines produced with group 8 “restored” serum, BALB/c T15 myeloma protein, and serum from B10.D2/o mice “restored” with mouse:mouse hybrid cells (group 1). No reaction occurred between antiserum against MuB1 and C5-deficient DBA/2 serum. Precipitation of T15 Fab idiotype specificity was quite separate and could easily be distinguished from the MuB1 specificity.

Absence of MuB1 in Extracts and Cultures of Chicken Tissues. Cultures of chicken spleen, kidney, and liver cells were established by procedures identical to those used for mouse cells. All cultures contained viable monolayers, but in no instance, did medium removed at intervals from the cultures restore hemolytic activity to C5-deficient mouse serum. Likewise, homogenates of chicken spleen, kidney, or peripheral blood cells failed to restore hemolytic activity to C5-deficient mouse serum and, by immunodiffusion, contained no MuB1 antigen.

DISCUSSION

C5 plays an important role in host defense since it not only is necessary for completion of the hemolytic complement sequence but also is a major source of a phlogistic peptide which mediates leukocyte accumulation and vascular permeability increases in various inflammatory states (10, 11). Our studies show that C5 activity can be restored to the serum of C5-deficient B10.D2/o mice by injection of hybrid cells composed of cells that do not produce C5 from C5-normal animals and splenic macrophages from C5-deficient mice. The restored C5 was functionally and anti-genically normal in that it had both hemolytic and chemotaxis-generating activity as well as immunologic identity with MuB1. The time-dependent appearance of C5 activity in both *in vitro* and *in vivo* experiments suggests that C5 synthesis was induced *de novo*

FIG. 6. Identification of MuB1 antigen in “restored” sera by double-immunodiffusion in agar gel. Antiserum against MuB1 was raised by inoculating A/He mice with BALB/c myeloma protein TEPC 15. Antiserum against MuB1 (anti-MuB1) was reacted against sera from mice inoculated 0, 3, 6, and 14 days before with B10.D2/o spleen:B10.D2/n kidney (group 1) or B10.D2/o spleen:chicken erythrocyte (group 8) hybrid cells or B10.D2/n spleen cells (group 6). Control antigens included serum from MuB1-positive DBA/1 and MuB1-negative DBA/2 mice and TEPC 15 (T15) protein.

in hybrid cells. Inhibition by puromycin of *in vitro* C5 evolution supports this conclusion.

There are several alternatives to explain the nature of the genetic defect in B10.D2/o mice and its correction by cell hybridization. If the C5 deficiency is due to a structural gene deletion, then production of C5 by the mouse:mouse hybrid cells (group 1) might easily be explained by a transfer of the C5 structural gene of the normal kidney cells to a regulator milieu in splenic macrophages that allows its expression. Inconsistent with this interpretation, however, are the results with the mouse:chicken hybrids (group 8). Here, if the chicken erythrocyte provided the C5 structural gene, chicken C5 would have been produced. Double-immunodiffusion studies (Fig. 6) suggest that both mouse:mouse and mouse:chicken hybrids produced proteins identical with each other and with normal mouse MuB1. Furthermore, cultures and homogenates of normal chicken tissues failed to yield anything with MuB1 antigenic activity. It can be argued that our anti-MuB1 reagent would not detect chicken C5 were it produced by the mouse:chicken hybrids, but were these cells producing chicken C5 it would still not explain their concurrent production of mouse C5. It is possible that the C5 locus is complex, however, and that the deficient mouse lacks not the whole locus but just a single component thereof. The deficient animal may produce nonfunctional, nonantigenic subunits, the assembly of which requires the missing gene product. Here either a mouse or chicken substance may suffice.

Expression of the mouse C5 structural gene may normally be repressed in all cells except splenic macrophages, which may possess a derepressor substance. Lack of this derepressor in B10.D2/o mice could account for their C5 deficiency. Cell hybridization could produce a dilutional or other physical effect whereby the repressor may have an altered affinity at its effector site and release some structural gene function without need for the derepressor. An alternative but similar possibility is that the splenic macrophages of B10.D2/o mice have a C5 repressor absent in normal mouse macrophages. Arguing against both these mechanisms is the failure of hybrid cells from group 3 (B10.D2/n kidney:B10.D2/n kidney), group 4 (B10.D2/o spleen:B10.D2/o spleen), group 5 (B10.D2/o spleen:B10.D2/o kidney), and group 11 (B10.D2/n kidney:chicken erythrocytes) to produce C5. Furthermore, were the C5 deficiency in B10.D2/o mice to depend on such a tenuously bound repressor substance, the C5 deficiency would more likely be relative than absolute. Colten and Parkman (16) showed that hybrid cells composed of peritoneal exudate cells from C4-deficient guinea pigs and HeLa cells, derived from a human neoplasm, synthesized human C4. These results were explained (17) by the elaboration, by the guinea pig cells, of a factor able to induce the synthesis of C4 by the HeLa cells. Were such a substance operative in our system, the hybrid cells composed of B10.D2/o macrophages and chicken erythrocytes would have produced chicken, rather than mouse, C5.

These experiments may provide a prototype for practical

genetic repair in humans. Most human deficiency states are only relative deficiencies. By 21 days after "restoration," our mice produced antibody to the newly synthesized C5 (4) because it had been a foreign protein to them. In the relative deficiency states of humans, however, no such antibody response would be expected. Complicating experiments in man is the fact that hybrids produced from the cells of two non-identical humans would express the histocompatibility antigens of both the deficient and the normal parental cells and thus would be rejected by either cell donor. It may be possible to select *in vitro* clones of hybrid cells that both synthesize the missing gene product and are histocompatible with the deficient recipient. Indeed, clones histoincompatible with the deficient recipient could be eliminated by a technique similar to that of Puck *et al.* (18), using antisera raised against the histoincompatible cell component of the hybrids. Particularly useful might be hybrids composed of deficient cells and normal human erythrocyte precursors. The normoblast, being nucleated, provides a cell analogous to the chicken erythrocyte and might, like the latter cell (19), not directly contribute membrane components to the hybrid cells.

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