Tissue Culture Demyelination by Normal Human Serum

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Serum from all of 20 normal individuals induced some degree of demyelination when applied to well-myelinated mouse cerebellum cultures. An intact complement sequence through C5 is required. Demyelinating activity was heat labile at 56°C for 30 minutes but was not destroyed at 50°C for 30 minutes (which inhibits properdin factor B and alternate complement pathway activation, but not the classic complement pathway). Sera from patients with agammaglobulinemia, C4 deficiency, or C6 deficiency all induced demyelination. Our results suggest that tissue culture demyelination results from nonimmunoglobulin activation of the alternate complement pathway and is not limited to sera from patients with neurological disease.

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Work by many investigators suggests that immunological processes may play a role in the pathophysiological mechanisms of multiple sclerosis (MS). One such finding is the observation that the sera obtained from some patients with MS can produce demyelination of myelinated rodent brain cultures (Table 1). In these reports it was noted that a proportion of sera from normal subjects and from patients with other disorders, such as amyotrophic lateral sclerosis, also could produce culture demyelination. In the course of studies designed to develop methods of observation of myelinated cultures that do not depend on morphological inspection alone [14], we observed that sera from almost all normal, non-laboratory-related individuals produced demyelination and swelling of neuroglia in our tissue culture system. Therefore, we undertook a study to attempt to elucidate the mechanisms that underlie human serum-induced demyelination in vitro. Our data suggest that such demyelination is mediated through the alternate complement pathway. An intact complement sequence through the fifth component of complement (C5) appears to be required.

Materials and Methods

Maintenance of Cultures

Slices of newborn mouse cerebellum were explanted and maintained on rectangular coverslips coated with reconstituted rat tail collagen and maintained in roller tubes for 15 days following their initiation. One culture was started per coverslip. Each roller tube, containing one coverslip, received 0.7 ml routine feeding medium consisting of Eagle's minimal essential media (MEM) with Earle's balanced salts (MA Bioproducts), supplemented with 0.05 units/ml of low-zinc insulin (Squibb Institute of Medical Research, New Brunswick, NJ), 25% fetal bovine serum, and glucose to bring the final concentration to 600 mg/ml. The osmolarity was adjusted by adding deionized water to achieve 280 mOsm. The medium was changed every 7 days, including the day before the experiment.

On the morning of an experiment, cultures were removed from the roller tubes and were placed in Maximow depression slides to allow selection of suitable cultures by light microscopy. Cultures with several hundred or more strands of myelin and an absence of necrotic areas were divided into groups and washed briefly in Earle's balanced salt solution with 600 mg/dl of glucose. Experimental media consisted of 75% Eagle's MEM with supplemental glucose (600 mg/ml), insulin (0.05 μ/ml), and 25% normal human serum. Each group of cultures was fed media containing either serum from one of 20 normal humans, or fetal bovine serum as a control. Slices of cerebellum were intermixed at the time of explant, so that specimens from several animals in a litter used as the source of tissues were intermixed through the group of cultures used for a particular experiment. After application of experimental or control media, the cultures were coded, intermixed, and interpreted blindly by two observers (D. H. S., M. C. M.). Cultures were routinely observed by light microscopy in the living state 20 to 24 hours after application of the experimental and control media.

The criteria used for recording changes in culture structure were as follows:

1. The appearance of many blebs along otherwise intact myelinated axons or a reduction in the number of intact my-

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Table 1. Percentage of Sera That Demyelinated Cultures

Researchers	MS	Controls and/or ONDs
Bornstein [2]	55 (active) 37 (all MS)	20
Hughes and Field [6]	84	23
Lamoureux and Borduas [8]	93	21
Lumsden [9]	100	24
Ulrich [15]	14	51
Wolfgram et al [16]	55	70

MS = multiple sclerosis; ONDs = other neurological diseases.

elinated axons (either or both changes warranted a probable demyelination designation)

2. A marked decrease in the number of intact myelinated axons or a total loss of myelinated axons (either change was recorded as definite demyelination)
3. Swelling of large neurons (recorded as a toxic reaction)

Preparation of Human Serum

Blood was collected from each of 9 laboratory-related and 11 non-laboratory-related individuals. The blood was allowed to clot at room temperature, and the tube was rimmed and spun in a refrigerated centrifuge at 2,000 rpm for 15 minutes. The serum was removed and stored at −70°C within 1 hour of collection.

Sera were absorbed with either rat brain acetone powder or mouse live acetone powder (Sigma Chemical Co, St Louis, MO) in a ratio of 2:1, v/v, at 37°C for 1 hour. The tubes were spun in a Beckman microfuge, and the supernatant was filtered through a sterile Millipore filter (0.22 μ). Unabsorbed sera were processed in the same fashion, in parallel.

Disodium-EDTA, 100 mM, or 100 mM of EDTA with 10 mM of $MgCl_2 \cdot 6H_2O$ (Mg^{++} - EGTA) was prepared in a phosphate buffer, pH 7.4. Sera were incubated for 2 hours at 37°C with either 5 mM of final EDTA to chelate both Ca^{++} and Mg^{++} or 5 mM of final Mg^{++} - EGTA to chelate Ca^{++}. Eagle's MEM without calcium was used to make up the experimental media.

Human sera were depleted of C3 in vitro by incubation with zymosan [12]. Zymosan, 4 mg, was incubated with 1 ml of serum at 37°C for 45 minutes, and the supernatant filtered through a sterile Millipore filter (0.22 μ). As a control, unabsorbed serum from the same individual was processed in parallel.

Sera were heated at either 56°C for 30 minutes to inhibit activation of both the classic and alternate complement pathways (C1, C2, properdin factor B) or at 50°C for 45 minutes to inactivate properdin factor B and inhibit complement pathway activation [11].

Sera deficient in the fourth component (C4) were obtained from two patients with hereditary angioedema and serum C4 levels 1% of normal [3]. These sera had normal levels of C1 and C3, and an intact alternate complement pathway. Serum from a patient with isolated deficiency of the sixth component of complement (C6) was also employed [4]. This serum had normal levels of C1, C3, C4, C5, and C7, as assessed by effective molecule titration [10] in our laboratory. Serum C6 level was 1% of normal. Agammaglobulinemic serum from a patient with severe congenital immunoglobulin deficiency was a gift of Dr Mary Anne South, Professor of Pediatrics, University of Texas School of Medicine, Lubbock, TX.

Results

Unaltered human serum induced at least some pathological demyelinating alterations in almost every instance (Table 2; Figs 1 and 2). In most cultures undergoing marked demyelination, swelling of neuroglia, and sometimes of granular neurons, preceded or accompanied changes in myelinated axons. Some sera induced swelling of both glial cells and neurons of the cerebellar roof nuclei, as well as demyelination, and were termed "toxic" (Fig 3).

Changes in the appearance of myelinated axons, neuroglia, or neurons were not produced by fetal bovine serum controls in any of the experimental situations, except in the experiments employing EDTA and Mg^{++}-EDTA (to be discussed). Each human serum was tested on a group of four cultures on one or more occasions. Control exposures were included on every experimental day. When results within a group of four cultures were inconsistent, that serum was retested. The possible reasons for inconsistency within a group of cultures exposed to a serum on a particular day include variation in culture thickness and variation in the starting condition of the culture (although all cultures were selected for being in excellent condition prior to being included in an experiment).

The kinetics of the changes induced by human serum were studied by exposing cultures to human serum for varying periods of time, washing with balanced salt solution supplemented with glucose, and refeeding with fetal bovine serum medium in Maximow depression slide assemblies. Exposure to serum for a period of time as short as 3 hours was sufficient to initiate the sequence of events leading to demyelination. In serial observations the first changes seen were swelling of neuroglia and the appearance of blebs along the otherwise smooth course of myelinated axons. The changes continued, reaching their maximum by approximately 18 hours. Decreasing the concentration of human serum (to 12% or 6%) resulted in a decrease in the amount of demyelination occurring.

The ability of human serum to demyelinate cultures was evident in both the presence and the absence of 10% guinea pig serum. When sera were heated to 56°C for 30 minutes, no demyelination occurred (Table 3; Fig 4). In those sera that induced cytotoxicity as well as demyelination, cell swelling was also prevented by heating to 56°C. The addition of 10% guinea pig serum to heated sera did not result in restoration of the ability to induce demyelination.

When sera treated with Mg^{++}-EDTA and EDTA

Table 2. Demyelination by Unaltered Normal Human Sera

Subject No.	No. of Cultures			
	Total Tested	Definite Demyelination	Probable Demyelination	No Demyelination
NONLABORATORY PERSONNEL				
1	4	4	0	0
2	4	3	0	1
3	4	2 (1)[a]	1	1
4	20	16 (1)	1 (1)	3
5	4	3 (1)	1	0
6	28	22 (1)	1	5
7	12	8 (1)	2	2
8	4	3 (1)	1	0
9	8	5 (4)	3 (2)	0
10	28	20	4 (1)	4
11	8	7	0	1
LABORATORY PERSONNEL				
1	8	8 (3)	0	0
2	4	2	2	0
3	7	7 (7)	0	0
4	4	4 (2)	0	0
5	8	5 (2)	3 (1)	0
6	4	3 (3)	0	1
7	4	3 (2)	0	1
8	4	4 (3)	0	0
9	4	3 (1)	0	1

[a]Parentheses indicate number of cultures showing generalized cytotoxicity.

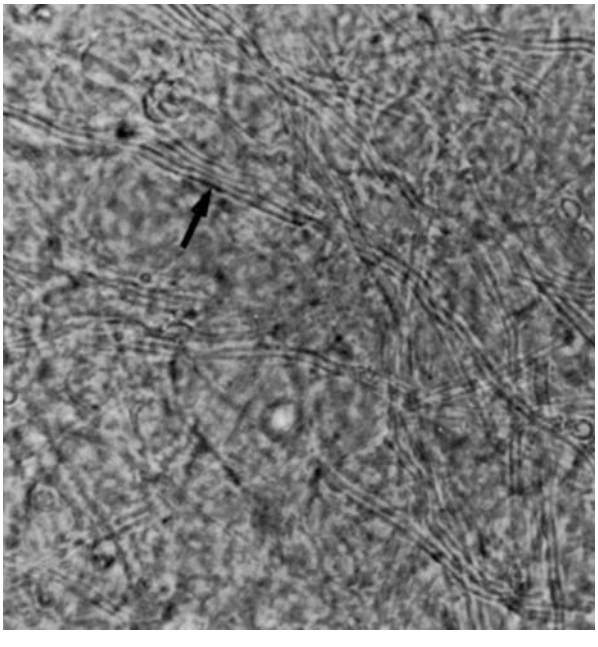

Fig 1. Newborn mouse cerebellum 15 days in culture; following 24-hour exposure to 25% fetal bovine serum. Note well-delineated myelinated axons (arrow). *(Unstained; bright field; ×600.)*

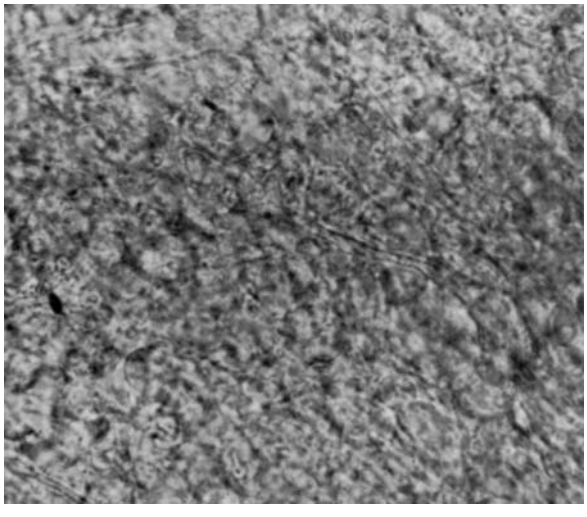

Fig 2. Newborn mouse cerebellum 15 days in culture; following 24-hour exposure to medium containing 25% unaltered normal human serum. Note dissolution and fragmentation of most myelin sheaths, leaving bare axons. (Unstained; bright field; ×600, before 20% reduction.)

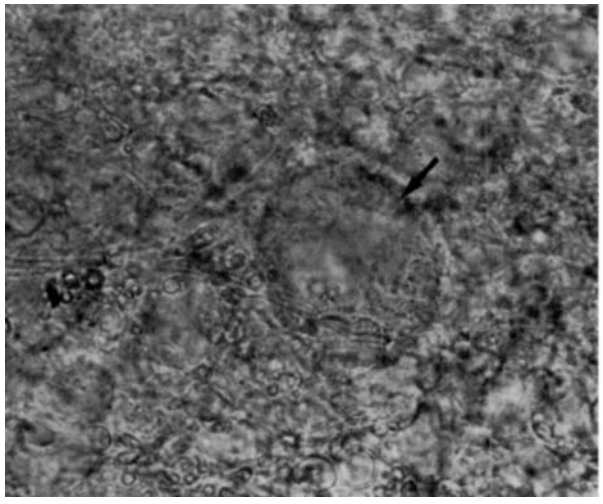

Fig 3. Similar culture to that in Figure 2 following 24-hour exposure to medium containing 25% unaltered normal human serum. Note swollen large neuron (arrow), *indicating "toxic" response as well as demyelination. (Unstained; bright field; ×600, before 20% reduction.)*

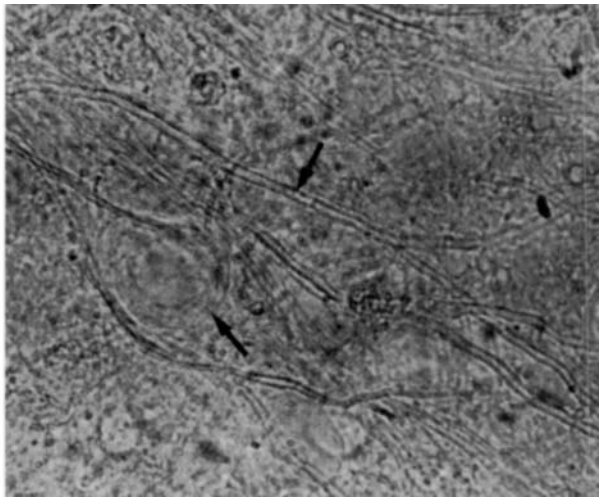

Fig 4. Similar culture to that in Figure 2 exposed for 24 hours to normal human serum that had been heated to 56°C for 30 minutes before use. Note intact myelinated axons, neuron (arrows). *(Unstained; bright field; ×600, before 20% reduction.)*

were employed, the architecture of the cultures was sufficiently distorted so as to make interpretation difficult. In those experiments the required calcium-free medium produced glial swelling and demyelination in the presence of fetal calf serum as well as with human serum.

Evidence for Involvement of the Alternate Complement Pathway

Human sera heated to 50°C for 30 minutes did not cause any change in the cultures (see Table 3). This maneuver inactivates properdin factor B, a crucial component of the alternate complement pathway. The classic complement pathway remains intact under these circumstances. In contrast, zymosan, which depletes sera of proteins necessary for alternate pathway activation [12], removed demyelinating activity from the human sera. Absorption of sera with either brain or liver powder did not affect the demyelinating activity of the sera.

A series of experiments was performed with plasmas obtained from individuals with an isolated congenital deficiency (Table 4). Sera from two individuals with C4 deficiency, one individual with agammaglobulinemia, and one individual with C6 deficiency all effectively demyelinated the cultures.

Discussion

The present studies indicate that the active molecule or molecules in normal human serum responsible for demyelination in the culture system used are heat sensitive. This observation suggests that the complement system was involved in the pathogenesis of the demyelination. Heating serum to 56°C results in inactivation of C1 and C2 of the classic complement pathway and properdin factor B of the alternate pathway [10, 11]. Serum heated to 50°C inactivates only properdin factor B, however. Similarly, treatment of human sera with zymosan, which depletes serum of properdin factor B and properdin factor D [12], also inhibits the demye-

Table 3. Effect of Complement-Depleted and Complement-Inactivated Sera on Demyelination[a]

Treatment of Sera	No. of Cultures			
	No. of Sera Tested	Definite Demyelination	Probable Demyelination	No Demyelination
Adsorbed with zymosan	5	0	1	22
Heated (50°C for 45 min)	5	1	4	16
Heated (56°C for 45 min)	5	0	0	15
Unabsorbed and unheated controls	5	15	2	7

[a]Sera were depleted of complement (C3) with zymosan; complement was inactivated by exposing sera to heat (56 or 50°C).

Table 4. Effect of Congenitally Deficient Sera on Demyelination

Sera	No. of Cultures		
	Definite Demyelination	Probable Demyelination	No Demyelination
Agammaglobulinemic	4	0	0
C4 deficient No. 1	2 (1)[a]	1	1
C4 deficient No. 2	3 (1)	1	0
C6 deficient	3	0	0

[a]Parentheses indicate number of cultures showing generalized cytotoxicity.

lination process. These studies, taken together, suggest that the alternate complement pathway is involved in the pathogenesis of the demyelination.

A further series of experiments was performed to attempt to determine which plasma proteins were required for demyelination (see Table 4). The ability of the reaction to proceed in agammaglobulinemia plasma is consistent with the involvement of the alternate complement pathway, which may be activated in the absence of antibody [13]. The ability of the reaction to proceed in the absence of the classic complement pathway protein C4 is also consistent with this concept. The capacity of C6-deficient serum to support demyelination argues for a requirement of only intact complement sequences through the C3 or C5 step. These studies thus suggest the involvement of complement in the demyelination of rodent brain cultures by normal human serum.

As described in Table 1, other investigators [2, 6, 8, 9, 15, 16] have also noted the induction of tissue culture demyelination by normal human sera, as well as by serum from a variety of non-MS neurological diseases. The proportion of sera capable of inducing demyelination has varied markedly among reported series. In an earlier and possibly relevant study, Bolande [1] showed most normal adult human sera to be cytotoxic to nonneural cell culture lines derived from both rodent and human sources. Neonatal cord serum was considerably less toxic. The toxicity was removed by heating the sera to 56°C for 30 minutes and was not restored by the addition of guinea pig serum.

Our experience raises two questions. The first is what factors led to our finding that virtually all normal human sera produce some degree of tissue culture demyelination. The second is what implications, if any, our observations have for the possible role of immunological alterations in the pathogenesis of MS.

Several factors may have led to our finding demyelination by normal human sera. First, our cultures were maintained in a roller tube system, which promotes spreading and flattening of the explanted fragments of mouse cerebellum. This produces cultures in which the myelinated axons are closer to the surface that is bathed by medium than they are in cultures maintained from the time of explant in Maximow double coverslip assemblies. As a consequence, penetration of serum components to the level of the myelinated axons may occur more readily in roller tube cultures. We considered the possibility that roller tube cultures may be more unstable than cultures developed in Maximow assemblies; however, we see similar results using cultures maintained from explant in Maximow assemblies. Conversely, heated human sera, and most normal sera of fetal calf, calf, rabbit, and bovine origin, do not induce demyelination in roller tube cultures. Transfer of cultures from roller tubes to Maximow assemblies may render cultures more susceptible to noxious factors; however, the same change in environment in the presence of the various inactivated human (or unaltered animal) sera did not produce demyelination. Finally, we included as demyelinated those cultures that also showed cytotoxic alterations in large neurons, because this more generalized cytotoxicity also appears to be mediated by the alternate complement pathway.

Other reports indicate a lack of evidence for immunoglobulin-mediated tissue culture demyelination by sera from patients with MS [5, 7]. Our results suggest that the culture demyelination observed by these and other workers may be the result of some other mechanism that activates the alternate complement pathway. This mechanism may or may not be the same in normal serum as in serum from patients with MS; however, we see no evidence at present that distinguishes tissue culture demyelination produced by unaltered normal human serum from that produced by unaltered serum from patients with MS.

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