Isolation of a Natural Cytotoxic IgM "Antibody" in Human Serum Sensitizing L Cells to Complement (42938)

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Abstract. **An IgM fraction of human serum was isolated and purified. A portion of this fraction firmly attaches to L cells' surfaces, which sensitizes these cells to the lytic action of low concentrations of serum C. It contains the natural cytotoxic "antibody" to L cells.** [P.S.E.B.M. 1989, Vol 191]

The natural cytotoxicity of human serum to a variety of cell strains has been recognized for some time (1, 2). This activity is universally present in the serum of pregnant women. Malignant or transformed cells are more susceptible than normal cells which are generally nonreactive. The rapidity with which cell death or lysis occurs has suggested that the cytotoxic reaction results from the action of serum complement (C) in conjunction with natural antibody, and experimental studies have supported this concept (3–7).

Studying the transformed and malignant murine cell lines—L cells, sarcoma 180, and Ehrlich ascites tumor cells—we have recently shown that the cytotoxicity is primarily dependent on a rapid attachment of an IgM "antibody" to the cell surfaces, which sensitizes the cell to the lytic action of low concentrations of serum C (8). Both the alternative and classical pathways must be intact for cytolysis to occur as both are involved in the reaction. The components of the system and their pathophysiologic role in the cytotoxic reaction was studied by immunologic, immunochemical, and immunocytochemical methods. The purpose of this experiment was to apply more direct biochemical methodology for the isolation of the IgM fraction of the serum, and further characterize the natural "antibody" residing therein.

Materials and Methods

Cell Cultures. The L cell (strain L929), a transformed murine fibroblast, was used in these experiments, as it epitomizes the reactions of all of the murine cells studied previously (8). The cells were serially propagated in Dulbecco's modified minimal essential medium supplemented with 10% neonatal calf serum.

Serum Pools. Serum pools were made from freshly drawn, clotted samples obtained from 10 to 15 normal pregnant women in the second and third trimesters. Gestational sera were used, as they were almost universally cytotoxic (8). Each serum was centrifuged at 15,000 rpm for 15 min at 4°C prior to pooling. Serum pools were either used immediately or snap-frozen in liquid N_2 and stored at −80°C, and then thawed at 37°C immediately prior to use. Long-term cold storage is inadvisable as cytotoxicity tends to be progressively lost (2).

Cytotoxicity Assays. *Trypan blue assay (8).* Cells were grown to confluence in Falcon 3001 plastic petri dishes (30 × 10 mm) containing 1–2 × 10^6 L cells. The cells were washed in physiologic buffered saline (PBS) and then inoculated with 1.5 ml of serum or serum reagent for 15 min at 37°C. Cytotoxicity was determined by treating the cell films with 0.3% trypan blue in PBS solution for 3 to 5 min at room temperature. The percentage of trypanophilic cells, indicative of the extent of cell death, was determined by counting 100 cells in a calibrated eyepiece reticle.

Cytosensitization assays. The cell films were washed free of growth medium with PBS and chilled to 4°C. They were then overlain with 1.5 ml of chilled serum pool and incubated for 20 to 30 min at 4°C. The serum was then aspirated and the cell films were washed three times with 2-ml volumes of chilled PBS. After this time, the cells showed no evidence of cytotoxicity at 4°C with trypan blue or following incubation at 37°C, when tested with trypan blue, but were unusually sensitive to the lytic action of small amounts of serum C. As such they were referred to as sensitized cells. After

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incubation of sensitized cells for 15 min at 37°C with fresh serum diluted 1/16, 70–100% of cells were trypanophilic. Unsensitized control cells were incubated at 4°C in PBS for 30 min and washed three times with 2-ml volumes of chilled PBS. They showed no trypanophilia after exposure to serum 1/16.

Cells sensitized in the same manner with the various fractions and reagents derived from serum (see below) were then exposed to serum diluted 1/16 as described above. The degree of cell death was expressed by the percentage of trypanophilic cells after exposure to the trypan blue reagent, and indicated the sensitizing activity of the serum fraction tested. Assays on unsensitized control cells were carried out simultaneously.

Serum Fractionations. Fresh toxic gestational serum pools were applied in 4-ml aliquots to Sephadex G200 columns (Pharmacia, 100 cm). The eluting buffer was PBS (pH 7.4) containing up to 0.5% NaN_3. Three-milliliter samples were collected in 160–170 tubes at a rate of 18 ml/hr. The UV absorbancies of each tube were read at 280 nm in a Gilford spectrophotometer. Three distinct peaks were produced, the first representing the void volume (Fig. 1). Tubes from the midportions of each of the three peaks were pooled, concentrated five times on a Minicon concentrator, and tested for cytosensitizing activity. From Peak 1 (void volume) tubes 18–23 were pooled, Peak 2 tubes 39–48, and Peak 3 tubes 63–72. Next, tubes 16–20, from the ascending slope of the void volume (peak 1), were pooled and then filtered through an Amicon XM300 filter, so that molecules with a M_r over 300 kDa were retained and concentrated four times over the original sample volume. This sample was expected to contain only IgM and macroglobulins.

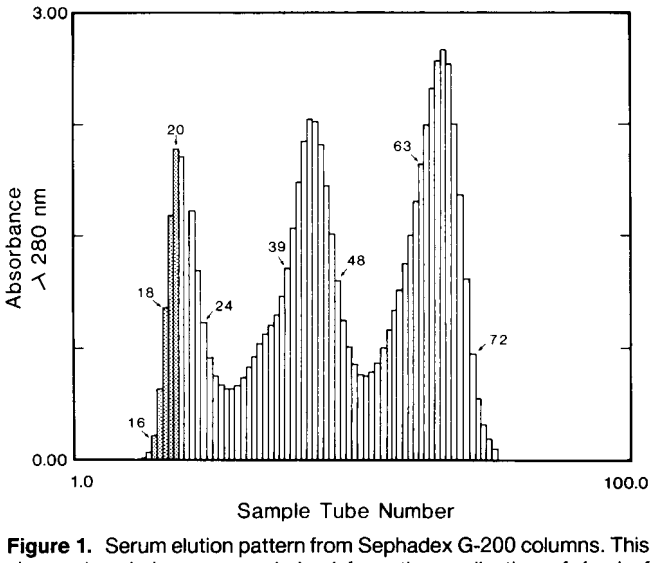

Figure 1. Serum elution pattern from Sephadex G-200 columns. This shows the elution pattern derived from the application of 4 ml of gestational serum to a Sephadex G-200 column. The first peak represents the void volume. Tubes 18–24 were pooled from the first peak, tubes 39–48 from the second peak, and tubes 63–72 from the third peak. Tubes 16–20 from the ascending slope of the void volume peaks were also pooled (shaded area). Sensitizing activity was limited to the pool from the void volume alone (18–24 and 16–20).

To remove α_2-macroglobulin, 6- to 10-ml samples of the prefiltration void volume pools were incubated at 37°C for 1 hr with rabbit IgG anti-human α_2-macroglobulin (Sigma) diluted 1/9 in PBS. The immune precipitate formed was removed by centrifugation at 10,000 rpm for 10 min. The supernatant was then filtered through an Amicon XM300 filter, concentrated four times over the original volume, and then tested for cytosensitizing activity.

Characterization of Serum Fractions. *Radial immunodiffusion.* Serum fractions were studied quantitatively by radial immunodiffusion for IgM, IgG, IgA, α_2-macroglobulin (Meloy) and albumin (Behring).

Polyacrylamide gel electrophoresis. Electrophoresis of serum fractions was performed on a Bio-Rad slab (Protean II) using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis system according to Laemmli (9). The gels were fixed and silver stained according to the Bio-Rad method.

Total protein determinations. Total protein of fractions was measured according to the bicinchoninic acid-protein assay method (Pierce). The ratio of IgM to total protein was calculated and compared with native serum. Serum fractions were tested for their ability to sensitize L cells to serum diluted 1/16, as described above. Sensitizing activity per unit of total protein was calculated and compared with whole serum.

Results

Sensitized cells show 95–100% trypanophilia (dead cells) after exposure to serum 1/16. Unsensitized control cells show 0% trypanophilia. This illustrates the amplification of C-mediated cytolysis by cell sensitization (Table I).

The factor in serum which sensitizes L cells to the cytolytic action of the small amounts of C present in serum 1/16 is confined to the void volume peak derived from a Sephadex G-200 column. The ascending portion of the void volume peak, when filtered and concentrated four times in an XM300 filter, was shown to have maximum cytosensitizing activity (90–100%). This four-time concentrated IgM fraction was thus used in all experiments. Doubling dilutions of the four-time concentrated IgM fraction shows diminishing cytosensitizing activity. All activity is lost at 1/8 dilution.

The four-time concentrated IgM fraction contains 60–120 mg/dl of IgM and is devoid of IgA, IgG, and albumin. Alpha$_2$-macroglobulin is present at about half the concentration of IgM. The concentration of IgM is close to that found in normal serum, but represents a 19-fold increase in the IgM/protein ratio. Elimination of the α_2-macroglobulin by specific immune precipitation does not affect sensitizing activity. The results of a typical experiment are embodied in Table I.

These findings are confirmed by gel electrophoresis

Table I. Cytosensitization of L cells by IgM Fraction of Serum

Cells sensitized with[a]	Protein concentrations (mg/dl)							Cytotoxicity of sensitized cells to serum 1/16 (% dead cells)
	IgM	IgA	IgG	Albumin	α_2-Macroglobulin	Total protein	IgM/protein	
Serum 1/1	140	145	1400	3800	420	4720	0.03	95
Four-time concentrated IgM fraction	88	0	0	0	48	152	0.58	90
Four-time concentrated IgM fraction + anti-α_2-macroglobulin	62	0	0	0	0	233	—	90
Unsensitized cells[b]	—	—	—	—	—	—	—	0

[a] Sensitized cells: incubated with serum or serum fraction at 4°C.
[b] Unsensitized cells (controls): incubated with PBS at 4°C.

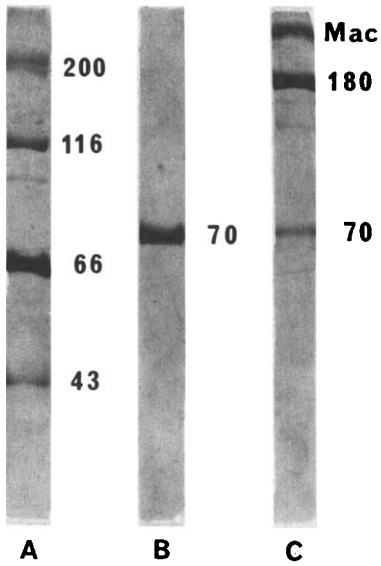

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of IgM fraction (Bio-Rad silver stain). Lane A shows the marker proteins. M_r in kilodaltons are indicated to the right. Lane B is the pattern produced by commercially purified IgM 1/100 (Cappel). Only the 70-kDa μ chain is seen. Lane C shows the pattern derived from the void volume pool (tubes 16–20) following ultrafiltration and concentration to 5×. The sample was diluted 1/10 before electrophoresis. The 180-kDa line represents monomeric IgM. The 70-kDa line is weaker. The line labeled Mac represents the macroglobulins.

(Fig. 2). The lines shown are degradation products of IgM and macroglobulins caused by the sodium dodecyl sulfate reagents' effect on these proteins. The commercially purified IgM is mainly represented by a 70-kDa silver precipitate, representing the μ chain of IgM. The IgM fraction that we prepared also shows a 180-kDa precipitate representing the monomeric form of IgM. The precipitates over 200 kDa are derived from macroglobulins. Elimination of the α_2-macroglobulin by specific immune precipitation does not affect the cytosensitizing activity.

Discussion

Our previous studies (8) showed that the sensitization of the murine cells to the lytic action of C was dependent on the binding of IgM to the target cells' surface. This was done by immunologic experiments which showed that:

i. The lytic action of serum was dependent on high levels of serum IgM.

ii. Adsorption of serum with cells at 4°C was associated with increased lytic reactivity to serum C and reduction of serum IgM.

iii. The lytic action of C could be blocked by treating sensitized cells with monoclonal anti-IgM, whereas anti-IgG, anti-IgA, and anti-C3 were without effect.

iv. Immunoperoxidase stains showed strong localization of IgM on the surface of sensitized cells, while IgG and IgA were not present.

The attachment of the IgM was tenacious. Numerous attempts to elute the bound IgM from sensitized cells using a variety of eluting agents, such as increasing ionic strength KCl urea, ethylene glycol, propionic acid, glycine-HCl and NaSCN, were unproductive or resulted in marked denaturation and loss of activity (unpublished data).

The method described here for preparing IgM from serum was necessary, as the IgM prepared by more conventional techniques and commercially purified IgMs showed little, or no sensitizing activity. We were able to obtain a reasonably pure IgM fraction which sensitized L cells to C to the same extent as normal serum, thus confirming our earlier immunologic studies. The active IgM fractions, while having about the same IgM concentrations as normal serum, showed a 19-fold increase in the ratio of IgM/total protein.

The term "antibody" is used tentatively in the absence of clearly defined antigenic receptor(s). It has been inferred from the tenacity of the bond between the IgM and the cell surface, and the concomitant fixation of complement, that a combination of antigen and antibody has indeed occurred (2, 5, 8). Evidence has been presented earlier that these antigens might be galactopeptides, resembling human blood group and oncofetal developmental antigens (5, 7). The latter seems more likely because of the increase in serum cytotoxicity occurring during pregnancy and extending into the postpartum period (7).

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