A Nonspecific Immunosuppressive Material Derived from Normal Bovine Serum[1] (37543)

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There is great need for clinically useful immunosuppressive agents. In 1958, Kamrin (1) discovered a fraction of normal serum which had immunosuppressive properties. Subsequent investigators (2, 3) have shown this material has no toxicity *in vivo* or *in vitro* at levels causing profound immunosuppression. The material is nonspecifically immunosuppressive. It is effective against a variety of immune responses elicited by a variety of antigens such as erythrocytes, serum and serum fractions. Effective immunosuppressive material may be syngeneic, allogeneic or xenogeneic to the recipient. The immunosuppressant has been found in fractions prepared from serum, plasma and tissues. Fractions prepared by different techniques (1–3, 17, 18) all had an alpha protein, most frequently designated α-globulin, as the major component.

It has been claimed that the serum-derived immunosuppressant interferes with an early immunologic event(s), possibly antigen recognition (2–4, 19). Certain models of the immune response (5, 6) have ascribed the function of antigen recognition to humoral natural antibodies. According to the model of Eisen and Karush (6) the ratio of the concentration of antigen to that of the corresponding antibody determines whether or not exposure to an antigen will result in the formation of antibodies, as well as the magnitude of the responses. Only the bimolecular complex (Ag-Ab) would lead to antigen recognition and immune stimulation. Thus the immunosuppressant derived from serum might complex with natural antibodies making them unavailable for formation of the immune stimulatory complex.

The present study was undertaken to seek an improved method of purification of the suppressive material. Experiments to characterize the material and probe its mode of action were also performed.

Materials and Methods. Bovine serum obtained from fresh blood was fractionated by an adaptation of the method of Mowbray and Hargrave (7). Charged DEAE-cellulose (from 30 g dry DEAE-cellulose) was equilibrated with 0.03 *M*, pH 5.0 acetate buffer. The cellulose was batch loaded with diluted serum (1 liter original volume) that had had euglobulins removed. The cellulose was batch washed and poured into a 2.5 $\times$ 40 cm glass column. One step elution was achieved with 0.5 *M*, pH 5.0 acetate buffer. This crude fraction was either neutralized and concentrated by ultrafiltration or lyophilized and stored at $-20°$.

To further purify crude fractions, the lyophilized material from 1 liter of serum was dissolved in 20 ml of distilled water. The solution was placed in boiling water for 9 min and cooled according to the method of Glasgow *et al.* (16). If required, coagulated protein was diluted and dispersed in a glass tissue grinder. Residue was removed by centrifugation and washed with distilled water.

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The supernatant fluid plus wash water were passed through an ultrafiltration membrane with exclusion above a mol wt of 10,000 (Amicon UM10). This filtrate was refiltered through a membrane with an exclusion above 500 mol wt (Amicon UM05). The retained material was concentrated to 20 ml and kept frozen until used.

Adult NIH white mice, in groups of 4 or more, were injected iv with 0.5 ml of immunosuppressant. Untreated mice served as controls. After 16–18 hr the mice were injected iv with 1×10^8 washed sheep red blood cells (SRBC). Four days after antigen injection, the mice were killed and their spleens were taken for enumeration of plaque forming cells (PFC). The plaquing procedure of Segre and Segre (8) was used with the following exceptions: 1% gelatin replaced BSA in the agar-medium mixture, only unsensitized SRBC were used and plates were incubated for 2 hr and 15 min.

In experiments on reversal of suppression, SRBC were coated with allogeneic anti-SRBC prior to injection. Specific mouse anti-SRBC antisera were produced as described by Henry and Jerne (9). The sera were titrated by hemagglutination (HA) and hemolysis (HL), both directly and by a Coombs-type antiglobulin procedure (10). Early immune serum (EIS) or late immune serum (LIS) was diluted in serial 10-fold dilutions prior to adsorption on to SRBC. Two milliliters of a 2.5% suspension of SRBC were placed in a 12×75 mm tube and centrifuged. The supernatant fluid was aspirated and 4 ml of serum dilution were added to the pellet. The tube was shaken, incubated at 37° for 30 min, shaken again and incubated at 4° for 16–18 hr. Prior to injection the cells were washed, counted and the dose adjusted to provide 1×10^8 SRBC.

Results. The immunosuppressive activity of crude DEAE-cellulose fractions was demonstrated in preliminary trials, confirming the results of others (2–3). Attempts were then made to further purify this material. Three procedures were employed: chromatography on Sephadex G-200, differential ultrafiltration on membranes with exclusion above 10,000 and 500 mol wt, and treatments intended to disrupt noncovalent intermolecular bonds (heat, acidification, 8 *M* urea, sodium dodecylsulfate). The results obtained are reported in Table I.

It is apparent that immunosuppressive activity was obtained only irregularly, both in the crude fractions from DEAE-cellulose and by further fractionation of these materials. When present, immunosuppressive activity appeared to be associated especially with the third peak eluted from Sephadex G-200. Heating the crude fractions at 100° for 9 min, followed by filtration through a UM10 membrane and concentration over a UM05 membrane increased the immunosuppressive activity of the crude fractions (crude fractions 10 and 12, Table I) even when no such activity was demonstrable prior to heat treatment (crude fraction 10, Table I). However, considerable activity was retained by the UM10 filter in some cases (crude fraction 15, Table I). Of 9 materials prepared by heating and ultrafiltration, 2 (preparations 12 and 18, Table I) were highly active. A pool of the five inactive preparations 17, 19, 20, 21 and 22 was concentrated 5 times over a UM05 membrane; the resulting preparation 23 had increased, but not statistically significant, activity (Table I).

Characterization of the heated, ultrafiltered preparations was carried out to a limited extent. The average protein content of 6 preparations, determined by the Folin-Lowry procedure, was 556 μg/ml or 11 mg protein/liter of serum. No correlation was found between suppressive activity and protein content as measured by this method. Since in each case a 20 ml crude fraction was derived from 1 liter of serum, comparisons were based on volume. The average carbohydrate content of 7 preparations, determined by the phenol–sulfuric acid test, was 19.3 μg/ml. RNase activity of the heated, ultrafiltered preparations was not measured, because of the limited quantities available. However, assay for RNase (11) of 11 crude DEAE-cellulose fractions (including fractions number 8, 10, 12 and 13, Table I) and 2 Sephadex G-200 third peaks (of fractions 4 and 7, Table I) yielded an average of 30 μg/ml. The average amount of RNase injected to each mouse was 11.7 μg (range 3.3–33.2 μg), well below the minimal dose of 70

TABLE I. Effect of Various Treatments and Fractionation Procedures on the Immunosuppressive Activity of Crude DEAE-Cellulose Fraction of Bovine Serum.

Crude fraction no.	Treatment or fraction[a]	Suppression[b] (%)
4	G-200 peak I, SDS, G-200	31
4	G-200 ascending limb of peak II, G-200	50[c]
4	G-200 descending limb of peak II, G-200	32
4	G-200 peak III, G-200	80[d]
7	G-200 peak III	3
8	Dialyzed	37
8	G-200 peak I	0
8	G-200 peak II	1
8	G-200 peak III	20
9	G-200 peak III	33[c]
9	G-200 peak III, G-200	0
10	None	0
Whole serum	Heat	0
10	Heat, G-25 peak I	23
10	Heat, G-25 peak II	36
10	Heat, filtrate UM10, retentate UM05	38
10	Acid, dialysate	0
12	None	26
12	8 *M* urea, filtrate UM10, retentate UM05	0
12	Heat, filtrate UM10, retentate UM05	90[d]
13	None	0
15	Heat, retentate UM10	56[d]
15	Heat, filtrate UM10, retentate UM05	33
17	Heat, filtrate UM10, retentate UM05	0
18	Heat, filtrate UM10, retentate UM05	96[d]
19	Heat, filtrate UM10, retentate UM05	0
20	Heat, filtrate UM10, retentate UM05	0
21	Heat, filtrate UM10, retentate UM05	31
22	Heat, filtrate UM10, retentate UM05	5
23[e]	Heat, filtrate UM10, retentate UM05	35

[a] Abbreviations used: G-200, G-25: Sephadex G-200 or G-25; peak I, II, III: first, second or third peak in order of elution from a Sephadex column, as measured by optical density at 280 nm wavelength; dialyzed: material retained after dialysis was tested; dialysate: material crossing the dialysis membrane was tested; SDS: 1% sodium dodecylsulfate; heat: material was heated at 100° for 9 min; acid: acidification to pH 3 by addition of 1 *N* HCl; UM10: ultrafilter membrane with exclusion above 10,000 mol wt; UM05: ultrafilter membrane with exclusion above 500 mol wt.

[b] Relative to the mean number of PFC for 31 control mice.

[c] Suppression significant at the 0.05 level (*t* test).

[d] Suppression significant at the 0.01 level (*t* test).

[e] This preparation was a pool of the heated and filtered preparations 17, 19–22, concentrated 5 times over a UM05 membrane.

μg reported to cause immunosuppression (7). Spectral analysis of 6 heated, ultrafiltered preparations from wave lengths 235 to 340 nm in 10 nm intervals revealed no distinctive features.

Serial 10-fold dilutions of highly active preparation 12 and minimally active preparation 15 were used in the suppression assay to determine the dose response. The results appear in Fig. 1.

The molecular weight of preparation 18 was estimated by use of molecular sieve chromatography through Sephadex G-50. Preparation 18 was highly active, eliciting 96% immunosuppression. The column void volume was determined with 0.15% blue

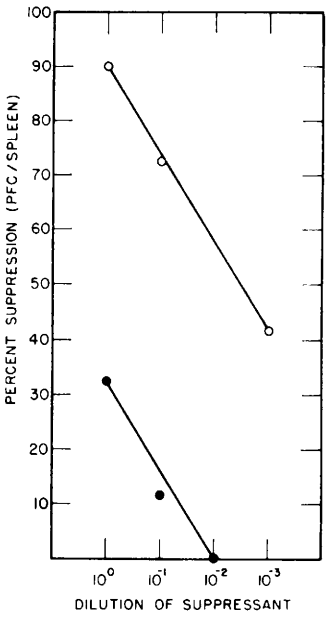

FIG. 1. Dose–response curves of preparations 12 (○—) and 15 (●—).

dextran 2000 eluted by a 0.15 *M* saline–borate buffer, pH 8. This was followed by preparation 18, lysine–vasopressin as low end marker and egg white lysozyme as high end marker, each eluted by 0.15 *M* saline–borate buffer. The dextran peak was located visually, preparation 18 was located by the Ninhydrin procedure, lysozyme by the lysoplate procedure (13), and vasopressin by bioassay in the rat (14). On Sephadex, over a considerable range, the elution volume (or tube number) is approximately a linear function of the logarithm of the molecular weight (15). When such a plot is constructed using vasopressin and lysozyme as markers of known molecular weight, preparation 18 corresponds to a mol wt of 8400 (Fig. 2).

Early immune serum (EIS) and late immune serum (LIS) were tested by direct and antiglobulin hemagglutination (HA) and hemolysis (HL). The sera were treated with 2-mercaptoethanol (2-ME) and direct HA and HL titrations repeated. The HA and HL titers of EIS were not significantly elevated by the antiglobulin procedure. Treatment of EIS by 2-ME abrogated direct HA and HL titers. Both HA and HL titers of LIS were greatly increased by antiglobulin treatment. Treatment of LIS by 2-ME caused a slight reduction of titer. Serial 10-fold dilutions of EIS and LIS were adsorbed onto the SRBC antigen before injection into mice which had been given immunosuppressant 16 hr earlier. The resulting number of plaque forming cells per spleen (PFC/spleen) in the experimental mice were converted to percentage of the PFC response of control mice given the same dose of suppressant and of untreated SRBC (Fig. 3). Decreasing dilutions of EIS from 10^{-5} to 10^{-2} caused increasing PFC responses of up to 245% of control level. Further decreases in the dilution of EIS caused a sharp drop in PFC response, finally reaching below control level. At a dilution of 10^{-5} LIS induced a PFC response below control level. At a dilution of 10^{-4} LIS induced an elevated response equal to EIS at the same dilution. Further decreasing dilutions of LIS induced decreasing PFC response finally reaching below control levels.

Discussion. Immunosuppressive activity was found associated with all 3 peaks eluted from Sephadex G-200 column, although it was more marked in peak III. It was, in part, retained by a UM10 ultrafilter mem-

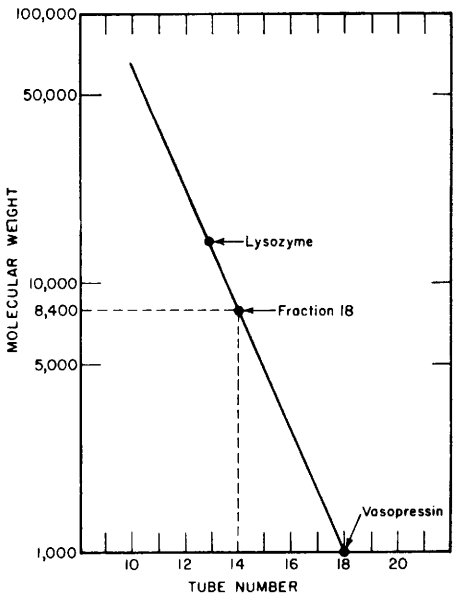

FIG. 2. Molecular weight approximation of preparation 18 on Sephadex G-50.

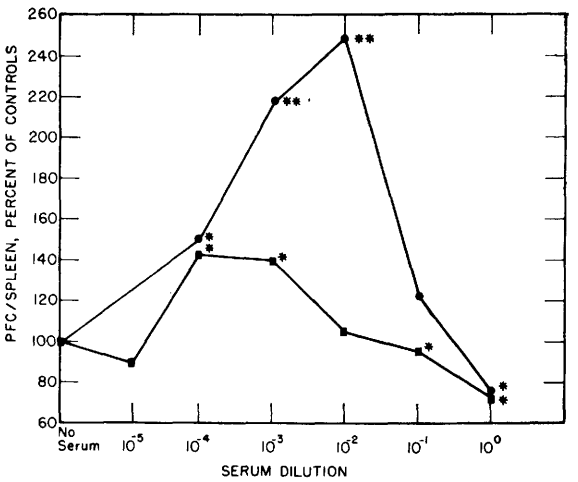

FIG. 3. PFC response of mice treated with suppressant and given SRBC coated with indicated dilutions of anti-SRBC from either early (EIS) or late (LIS) mouse immune serum. The response is expressed as the percentage of the number of PFC/spleen found in control mice given suppressant and uncoated SRBC. Points represent the average of 4 determinations. Single and double asterisks indicate points differing from control value at the 5% (*) and 1% (**) levels of significance. (●—): EIS; (■—): LIS. "No serum" indicates the PFC response of control mice, which is 100% by definition.

brane, which is impermeable to molecules of 10,000 or greater mol wt. On the other hand, at least a portion of the immunosuppressive factor crossed the UM10 membrane, and its molecular weight was estimated at 8400 by chromatography on Sephadex G-50. To reconcile these contrasting findings, we assume that the active principle is a small molecule capable of binding larger macromolecules. This would explain why immunosuppressive activity has often been attributed to an α-globulin (2–4). The apparent effectiveness of the heat treatment in the purification procedure is probably attributable to disruption of noncovalent bonds with release of the active principle from the macromolecules to which it is bound.

The variability in immunosuppressive activity of crude DEAE-cellulose fractions prepared in identical manner from the same starting material has been noted by others (2–4, 7). This is probably related to the ability of the active principle to bind other molecules, many of which may be discarded during various preparatory procedures. However, even crude fractions of questionable activity may yield highly active preparations upon heating and concentration (fraction 12, Table I). Concentration of apparently inactive treated preparations may yield some immunosuppressive activity (preparation 23, Table I). The similar slope of the dose–response curves for highly active preparation 12 and marginally active preparation 15 (Fig. 1) suggests the presence of the active principle even in preparations which do not result in statistically significant immunosuppression with the assay used.

Glasgow *et al.* (16) also found that the immunosuppressant is heat stable and dialyzable. Ultracentrifugal analysis yielded an $S_{20,w}$ value of 0.6, equivalent to 7000–10,000 mol wt. Our estimation of the molecular weight of preparation 18 (Fig. 2) is only an approximation. Accuracy was reduced by the use of only 2 markers. Also, the suppressant eluted near the exclusion limit, increasing the error. In spite of these shortcomings, the observed mol wt of 8400 is in good agreement with the finding of Glasgow *et al.*

Heated, ultrafiltered preparations contained protein and some carbohydrate. There was no correlation betwen protein concentration and immunosuppressive activity, suggesting the presence of considerable amounts of impurities even in the most active prep-

arations. RNase activity of the crude DEAE-cellulose fractions was about 1/7 of the reported minimal immunosuppressive dose of this enzyme (7). We conclude tentatively that the active principle is a peptide.

The mechanism by which this material effects immunosuppression is unknown. Several investigators expressed the view that it interferes with antigen recognition (2–4). Antigen recognition by humoral, natural antibodies has been postulated in a model proposed by Eisen and Karush (6). We reasoned that if antigen recognition is effected by humoral natural (and immune) antibodies, and if the serum-derived suppressant interferes with antigen recognition, such interference may occur through binding of humoral antibodies by the suppressant. Bound antibodies may then become unable to specifically bind and recognize the corresponding antigens.

We therefore attempted to overcome the immunosuppression by administration of antibodies from mouse immune serum adsorbed onto the antigen, SRBC. EIS contained specific IgM nearly exclusive of specific IgG. This is indicated by the lack of enhancement of serologic titers by anti-mouse globulin and the abrogation of the titers by treatment with 2-ME. In contrast, LIS showed marked enhancement of titers by antiglobulin and a slight reduction of titer by 2-ME. This indicates that anti-SRBC antibodies in LIS were mostly but not exclusively IgG.

The results of administration of SRBC coated with mouse antibodies (Fig. 3) indicate that immunosuppression was reversed by antibodies contained in EIS and, to a lesser extent, in LIS. The greatest PFC response was obtained with EIS at a dilution of 10^{-2}. Since EIS appeared to contain exclusively IgM antibodies the effect must be ascribed to IgM antibodies. This is in agreement with the findings of Henry and Jerne (9). In contrast to these findings, more concentrated EIS failed to reverse suppression and may even have contributed to it. Such a result would be predicted by models of the antibody-mediated regulation of the immune response, such as that of Eisen and Karush (6), which postulate an optimal antigen:antibody ratio for immunogenicity, with immunosuppression at ratios either greater or smaller than optimal. However, the contribution of undetected contamination of EIS with small amounts of immunosuppressive IgG antibodies cannot be entirely ruled out.

Reversal of immunosuppression was also observed with LIS, although the effect was less pronounced. LIS contained predominantly IgG antibodies, but the data suggest that IgM antibodies were also present. It is therefore not clear whether reversal of immunosuppression by LIS was due to IgG antibodies or to contaminating IgM antibodies. On the other hand, the suppressive effect of concentrated LIS should be ascribed to IgG antibodies, in agreement with the findings of Henry and Jerne (9) and others who have reported that IgG antibodies are more effective than IgM antibodies as immunosuppressants.

Summary. An improved method of purification of a potent, nonspecific immunosuppressant from normal serum is described. The suppressive agent was shown to be heat stable and filterable through a membrane with exclusion above 10,000 mol wt. The active molecule appears to bind to larger molecules from which it is released by heating. Preliminary data suggest that the active principle is a peptide with a mol wt of approximately 8000.

Immunosuppression was reversed by administration of antigen-antibody complexes, rather than antigen alone. Antibodies of the IgM class were more effective than IgG antibodies in reversing immunosuppression. Experiments on reversal of immunosuppression suggest that the suppressive agent may interfere with antigen recognition, which is postulated to be a function of the concentration of preformed, circulating, specific antibodies.

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