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The Depression of the Immune Response by Serum Protein Fractions

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Summary. A number of serum protein fractions were tested for their ability to suppress the immune response of rats injected with washed sheep cells. Significant depression of the immune response was produced by human serum proteins, and by bovine α-globulin fractions. The mechanism of this effect is at present not clear.

INTRODUCTION

There is very great interest at the present time in the mechanism of the immune response of the body to foreign protein, and in the various factors which influence this.

In 1958, Kamrin reported that α-globulins derived from pooled rat serum injected into rats in parabiotic union was effective in prolonging the life of the animals and in obtaining successful unions. Later papers (Kamrin, 1959, 1960) showed that a number of protein fractions, particularly α_1- and α_2-globulins were effective in promoting successful skin homografts some of which lasted more than a year.

Further investigations of a similar type were carried out by Mowbray (1963a, b, c). This author prepared an α-globulin fraction of ox serum, and showed a striking reduction in the antibody response to injected red cells. There was also a prolongation of the life of homografts, applied to treated animals.

The precise physiological function of the α-globulin fractions of serum is at present not understood, and the discovery by Darcy (1955) of an α-globulin fraction of serum which is prominent during pregnancy, rapid growth, and malignant disease is of special interest. This work suggested the possibility that there might exist a physiological mechanism for the suppression of the immune response under conditions in which this response could be undesirable. It would thus be of some importance to establish whether the α-globulin components isolated by Mowbray and the protein fraction of Darcy (1960) contained a common factor.

The present experiments were undertaken to test the effect on the primary immune response, of various protein fractions, including the α-globulins of Mowbray (1963c) and Darcy (1960).

METHODS

Washed sheep cells were used as antigen for the stimulation of the primary antibody response in rats. The immune response was measured by the titre of sheep cell haemolysin appearing in the serum during the subsequent 14 days. In early experiments, the sheep cells were given intravenously, but later they were given intraperitoneally. The protein

studied was usually injected intraperitoneally daily during a period of 7 days prior to the administration of the sheep cell antigen.

Fresh sheep's blood was obtained from the abattoir, the cells centrifuged, and the supernatant plasma removed. The packed cells were washed three times with physiological saline and suspended in saline to give a final haemoglobin concentration of 0·33 g/100 ml (approximately 1 per cent by volume).

Intravenously into a tail vein, or intraperitoneally 1·0 ml of this suspension was given to white Wistar rats. The blood samples required for measurement of the haemolysin titre were collected by capillary tube from the tail, allowed to clot, and centrifuged to separate the serum, the latter being stored in the frozen state if the assay were not performed immediately.

Assay of the immune response

Sheep cells for the assay were prepared by washing three times in physiological saline as described above, and once with 0·004 M veronal buffer pH 7·2 in physiological saline containing 1·7 mM magnesium chloride and 0·025 mM calcium chloride. The cells were suspended in the same veronal buffer to give a haemoglobin concentration of 0·33 g/100 ml.

Doubling dilutions of the serum sample in physiological saline were set up, the washed sheep cells added (0·05 ml) and the mixture incubated at 37° for 30 minutes. Fresh guinea-pig serum absorbed with sheep cells (0·05 ml of a 1 : 10 dilution) was then added, shaken, and the mixture returned to the water bath at 37° for a further 30 minutes, shaking at 15 minutes, and again at 30 minutes. The last tube showing complete haemolysis was taken as the titre. A standard haemolytic serum was always included with each assay.

Preparation of protein fractions for injection

When whole human serum was used, this was obtained by pooling small amounts of fresh serum from blood donors or from healthy pregnant women. Whole serum from a single normal individual was also investigated.

Various commercially available protein fractions were used in some of the experiments. Albumin from normal human serum was obtained from the Cutter Laboratories, Berkeley, California. The γ-globulin was prepared by the Commonwealth Serum Laboratories, Melbourne, Australia. Cohn fractions III (β-globulin), IV (α and β) and VI (glycoprotein) were obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio.

The γ-globulin fraction of Mowbray (1963c) was prepared from ox serum essentially as described by this author, using Whatman DEAE-cellulose. In some preparations, the ox serum was fractionated by a column, and on other occasions, batchwise treatment with the DEAE-cellulose was employed. The adsorbed protein was washed with 0·03 M acetate buffer, pH 5, and the three fractions (IA, IB, IC) eluted with 0·1, 0·2 and 0·5 M acetate buffer, as described by Mowbray (1963c).

An α-globulin fraction was also prepared by a similar technique to that described by Darcy (1960). To one volume of ox serum, was added at room temperature one-fifth volume of 25 per cent salicylsulphonic acid slowly with continuous mechanical stirring. The precipitate was centrifuged down, and the supernatant fluid decanted, and cooled to 0°. Four volumes of ice cold acetone were added with stirring, the resulting precipitate was centrifuged down, and washed twice with cold acetone at 0°. It was dried in a vacuum desiccator. For use, it was dissolved by dialysing against 0·15 M disodium phosphate solution (fraction IIA).

A commercial preparation of the glycoprotein 'Fetuin' was obtained from the Colorado Serum Company (4950 York Street, Denver, Colorado, U.S.A.).

The dosage of the various α-globulin fractions was usually 40 mg/day, but smaller amounts were employed in a few experiments.

When whole serum or commercial albumin was used, the dosage levels were higher; 120–240 mg/day of whole serum protein was given, and up to 500 mg/day of albumin.

The cortisone employed was cortisone acetate 25 mg/ml (Cortelan Glaxo) given intramuscularly in dosages varying from 0·2 to 0·5 ml/day.

Statistical method

The haemolysin response on the 7th day after the sheep cell injection was expressed as the negative logarithm to the base 2, of the titre.

Means, standard deviations and the significance of differences between the control series and each experimental group were calculated. For the latter, Student's *t*-test was used (Fisher, 1950).

RESULTS

A. THE NORMAL RESPONSE

After the intravenous or intraperitoneal injection of 1·0 ml of washed sheep cells into a rat that has not had previous contact with these cells, a haemolysin develops which rises to a titre ranging from 1 : 500 to 1 : 8000 by the 5th to the 7th day, falling thereafter, at first rapidly then more slowly, the titre at 20 days still being of the order of 1 : 100 to 1 : 500.

In general when the cells were injected intravenously the peak titre was on the 5th to 6th day but after intraperitoneal injection the maximum titre was on the 7th day. The maximum haemolysin response also tended to be a little lower after intraperitoneal injection. In most of the experiments, the titres were measured on the 7th and on the 14th day after the sheep cell dose, but on some occasions only the 7th day titres were determined.

The initial titre of sheep cell haemolysin before the antigen stimulus was usually less than 1 : 16. In a few animals greater initial haemolysin levels were observed, but this did not appear to affect the subsequent peak titre in any unusual way.

In general two control animals were included with each experimental group, so that the control curves represent titres obtained at different times and with different batches of sheep cells throughout the whole experimental period.

The mean reciprocal 7-day titre of the group injected intraperitoneally was 1024, the sixteen values obtained with this group ranging from 512 to 8192.

B. THE EFFECT OF WHOLE SERUM INJECTIONS ON THE IMMUNE RESPONSE

Preliminary experiments showed that no diminution in the immune response occurred as a result of protein injections given following the sheep cell antigen dosage, and it was found that the most effective dosage schedule was an intraperitoneal injection of the protein preparation daily 7–11 days before the antigen. This method of administration of the protein was adopted as the routine procedure in most of the subsequent experiments. When whole serum was used the dosage employed was 2·0 ml daily given by intraperitoneal injection.

There was a variable reduction in the haemolysin titre, which in a few experiments amounted to total suppression of the primary immune reaction.

There was no significant difference between the effect of the serum of pregnant women and non-pregnant pooled serum.

In one experiment 2·0 ml of double strength protein was given, but the subsequent titre was no lower than with the normal protein dosage.

The mean 7-day titre of twenty-six rats of this group given intraperitoneal protein 7–11 days before the sheep cell injection was 1 : 200, values ranging from 1 : 8 to 1 : 2048.

This reduction in the mean titre is significant, $t = 4{\cdot}6$, $n = 42$ (Table 1), where n is the total number of animals in the control plus test series.

C. THE EFFECT OF MISCELLANEOUS PROTEIN FRACTIONS

The various protein fractions used were examined by paper electrophoresis, by electrophoresis in acrylamide gel, and by immunoelectrophoresis.

Fractions IA, and IB appeared as single α_2-globulin components on paper electrophoresis while IC showed a rather ill-defined double band in the same area. Acrylamide gel electrophoresis showed each of these fractions to be multiple, and immunoelectrophoresis of a pooled preparation of IA, IB and IC showed a very large number of components, at least 14 (Fig. 2a).

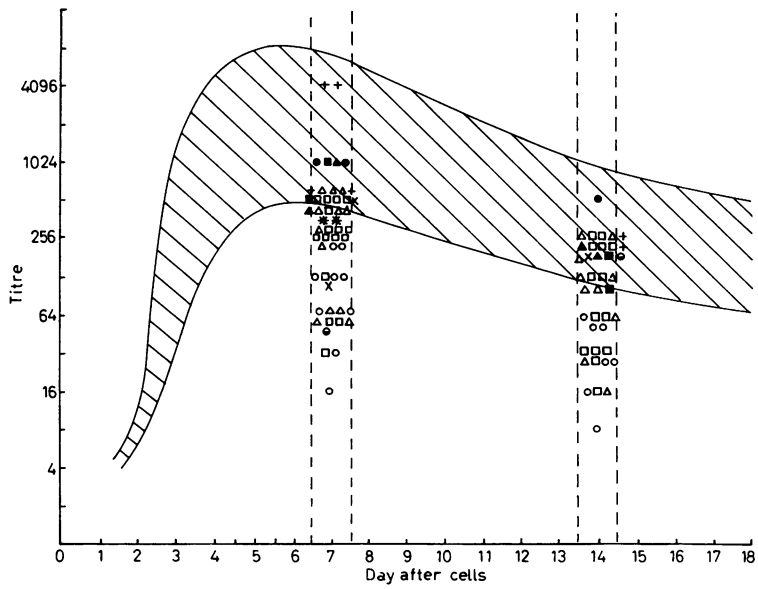

FIG. 1. The effect of intraperitoneal injections of various serum protein fractions. The figure shows the effect on the serum haemolysin titre of injections of various serum protein fractions. (The cross-hatched area indicates the range of normals.) □, IA; △, IB; ○, IC; +, IIA; ■, IIIB; ▲, IV; ●, VI; ×, albumin; ⊖, γ-globulin; *, Fetuin.

Fraction IIA appeared on paper electrophoresis to consist of a single component with the mobility of an α_1-globulin. By acrylamide gel electrophoresis it showed three well defined components, with other faint bands, and on immunoelectrophoresis there were two recognizable fractions (Fig. 2a).

Comparison by double diffusion in agar gel of a pooled preparation of IA, IB, IC obtained from human serum with fraction IIA also prepared from human serum, using

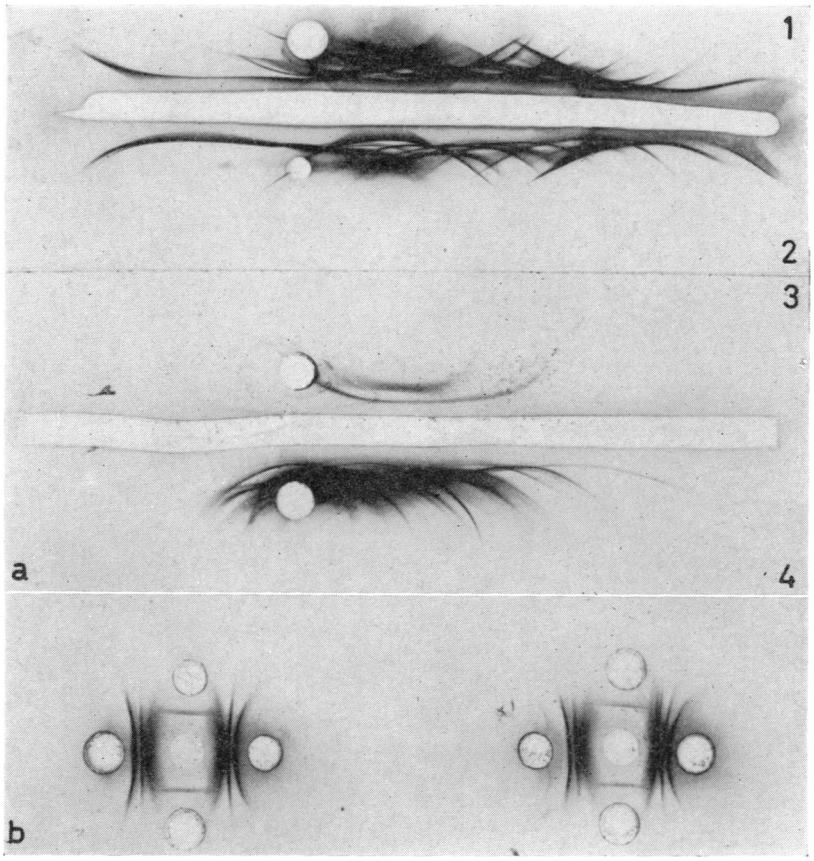

FIG. 2. (a) Immunoelectrophoresis on agar gel of protein fractions. (1) and (2) are normal human serum proteins; (3) is the pattern obtained from fraction IIA, prepared from human serum; and (4) is the pattern derived from a pooled preparation of IA, IB and IC, obtained from human serum (Pasteur Institute anti-serum). (b) Comparison of human serum protein fractions IA, IB, and IC with fraction IIA, using a micro version of the Ouchterlony double diffusion agar gel technique. There is no correspondence between either of the fractions IIA and any component of the group IA, IB, IC.

Pasteur Institute anti-human antiserum, showed no coincidence of the precipitin lines of IIA with any of those of IA, IB, IC (Fig. 2b).

The commercial Cohn protein fractions III, IV, VI and Fetuin, all contain globulins of the α_1 or α_2 type.

Fig. 1 and Table 1 summarize the effect of these various protein fractions on the haemolysin titre.

With fractions IA, IB, IC, the dosage was 40 mg/day, the mean titres obtained being 1 : 256, 1 : 256 and 1 : 64 respectively (Table 1). This reduction in the mean titre is significant (t = 5·7, 4·9 and 7·3 respectively), a result in general agreement with Mowbray (1963c) who found fraction IC the most effective. In our experience, however, none of these fractions induced as striking a fall in antibody response as that found by Mowbray (1963c), and a single dose of protein given 2 hours or more before the sheep cells was not effective in our hands.

TABLE 1

SUMMARY OF THE RESULTS

Dosage and type of protein given	No. of animals	7th day haemolysin response ($-\log_2$ titre)			Standard error of mean	Difference between means of experimental and control group	Significance of the difference between the means
		Range	Mean	Standard deviation			
Control group	16	9–13 (1/512–1/8192)	10·2 (1/1024)	±1·0	0·31		
2·0 ml of pooled normal human serum daily 7–11 days before sheep cells	26	3–11 (1/8–1/2048)	7·5 (1/200)	±2·2	0·80	2·7	$P<0·001$ ($n = 42$, $t = 4·6$)
40 mg of fraction IA daily 7 days before sheep cells	16	5–9 (1/32–1/512)	7·8 (1/200)	±1·3	0·46	2·4	$P<0·001$ ($n = 32$, $t = 5·7$)
40 mg of fraction IB daily 7 days before sheep cells	12	6–9 (1/64–1/512)	7·8 (1/200)	±1·5	0·53	2·4	$P<0·001$ ($n = 28$, $t = 4·9$)
40 mg of fraction IC daily 7 days before sheep cells	9	4–8 (1/16–1/256)	6·4 (1/80)	±1·5	0·60	3·8	$P<0·001$ ($n = 25$, $t = 7·3$)
40 mg of fraction IIA daily 7 days before sheep cells	4	9–12 (1/512–1/4096)	10·5 (1/1500)	±1·5	0·47	0·3	$P = 0·7$ ($n = 20$, $t = 0·45$)
Cortisone acetate 5–12·5 mg 4–7 days before sheep cells	7	3–11 (1/8–1/2048)	8·1 (1/256)	±3·2	1·10	2·1	$P<0·001$ ($n = 23$, $t = 4·4$)

The 7th day haemolysin response is represented by the negative logarithm to the base 2 of the titre. The actual titres are recorded in parentheses.

The glycoprotein fraction IIA, Fetuin, and the commercial fractions III, IV and VI showed no significant reduction in the titre of haemolysin in the treated animals.

There was a slight reduction in haemolysin titre produced by large dosages of albumin and γ-globulin.

D. EFFECT OF CORTISONE ON THE IMMUNE RESPONSE

Injections of cortisone following the sheep cell dosage had no effect in suppressing the primary response. Injections given for a number of days before the antigen did however have a depressing effect on the antibody production, an experience similar to that of Berglund (1956). As with the experiments using protein, however, the effect was variable and unpredictable, only three out of seven animals showing a reduction in the titre of haemolysin.

E. THE EFFECT OF A SECOND INJECTION OF SHEEP CELLS IN ANIMALS WHO HAD PREVIOUSLY BEEN TREATED WITH α-GLOBULIN

In order to try to define the length of time during which a depression of the immune response might continue after the protein treatment ceased, a second injection of sheep cells was given 17 and 14 days after the first antigen challenge to rats that had been treated with protein fraction IA for 7 days before the first sheep cell injection.

The results showed a slight reduction in the haemolysin titre 7 days after the last protein injection (mean titre 1 : 256), but a scarcely significant reduction after 14 days (mean titre 1 : 512).

DISCUSSION

Although the assay system in these experiments is very different from that of Kamrin (1958, 1959) who used the survival of parabiotic rats, and of skin homografts as a measure of the effectiveness of various protein fractions, the results obtained are similar. This author found some degree of activity from injections of whole serum protein, and from treatment with a number of different serum protein fractions. His most active serum component (fraction VI) secured homograft survival in 42 per cent of the animals tested, and other protein preparations were very nearly as good as this. He also found that the timing of the protein injection in relation to the antigen stimulus was important, and some of his experimental results suggested that pre-treatment was the most effective in the experiments with parabiotic union (Kamrin, 1958). The dosage schedule which he employed in later experiments (protein fraction 4–15 mg on days 0, 3, 6, 9, 12) was however ineffective in our hands.

Mowbray (1963c) reported a striking reduction in antibody response, using a variety of assay systems in both rabbits and rats, when a single injection of bovine fraction 1C was given 2 hours before the antigen challenge. In our experiments such a dose did not produce a significant reduction in haemolysin titre, though there was a measurable reduction in titre if the protein were given for some days before the sheep cell injection. Only in a few animals was the fall in titre considerable.

None of the protein injections given could be relied upon to produce a depression of the primary immune response, though on the average there was a significant reduction in antibody titre with a wide range of proteins tested.

Liacopoulos and co-workers in a series of papers published during the period 1962–65 and recently reviewed by him (Liacopoulos, 1965) have shown complete suppression of the immune response to a second antigen by the injection of large doses of one antigen (approximately 1 g/kg of body weight/day), given daily 7–18 days prior to the second antigen. It is possible that the mechanism of the depression of the immune response which we obtained is similar to that occurring in the experiments of Liacopoulos (particularly in the case of the injections of whole serum) but certain differences make it appear likely that different mechanisms may be involved. We found 500 mg/day of albumin was less effective than 40 mg/day of fraction IC, whereas 40 mg/day of fraction IIA and of Fetuin had no apparent effect at all.

If blockade of the antibody production were the explanation, it might be expected that the foreign protein would be most effective if given at the same time as the sheep cells, but this is not the case.

A strong argument against the competitive interference explanation is the observation by Mowbray (1963c) and Kamrin (1958) that homologous serum protein fractions were as effective as heterologous proteins in reducing the immune response. In a recent paper Thompson and Fishel (1965) show that fresh tissue protein from mice will inhibit the primary immune response of groups of mice of the same strain, an observation which also speaks against blockade of antibody production.

It is an interesting experimental fact that variations between individual animals in the effect of the injected protein component on the antibody response is far greater than the variability between individuals of the control group. This suggests that although there is a protein effect, more evident with some fractions than others, the suppression of the immune response involves some factor or factors other than the protein dosage alone. This is supported by the variable effect of cortisone, which was used without additional protein injections.

It is therefore clear that no adequate explanation is available at present for the effectiveness of protein fractions in reducing the primary immune response. Since the protein fractions used are complex, further purification may isolate a single protein or a protein bound factor with much higher activity.

Comparison by the Ouchterlony technique of the protein fractions IA, IB, IC and IIA show that these do not have detectable common components. There is thus no evidence that the protein fraction observed by Darcy (1955) which is increased in malignant disease, rapid growth, or pregnancy has any relationship with the protein fractions which depress the immune response. This is confirmed by the absence of activity observed in fractions IIA, and the observation that pooled pregnancy serum was no more effective in lowering the antibody response than non-pregnant human serum.

The existence of a system controlling the antibody response still remains as a possibility, though it would not seem to be related to the protein component of Darcy (1955).

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