Immunology, 1966, **11**, 413.

Further Studies on the Preparation of the Immunosuppressive Alpha$_2$ Protein Fraction from Serum and its Assay in Mice

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(*Received* 14*th February* 1966)

Summary. The method for the assay in mice of the α_2 glycoprotein fraction of serum with immunosuppressive activity is described. The crude fraction has been shown to be effective in mice only with intravenous injection. When an antigen which is rapidly cleared from the blood is given, a single injection of the protein fraction given 10–20 hours before the exposure to antigen will inhibit the immune response in most animals. Time intervals outside this range are associated with a diminished or absent immunosuppressive effect.

The protein fraction does not show activity immediately after preparation, or after reconstitution of freeze-dried material. Within 40 days, however, the activity is restored by storage alone, either frozen or at 4°. The presence of ribonuclease in the fraction is demonstrated, and its possible significance in relation to the immunosuppressive effect is discussed.

INTRODUCTION

In 1963, a fraction of serum, known as 'Fraction C', which on intraperitoneal injection, was capable of inhibiting antibody production in rats and rabbits was described from this department (Mowbray, 1963a). The crude fraction was found to contain a ribonuclease (Mowbray, 1963b). Since then there have been both successful and unsuccessful attempts to repeat this observation. Two failures to obtain activity have been reported (Davis and Boxer, 1965; Spiegelberg and Weigle, 1964). It has been suggested that the immunosuppressive component is labile and that it may be a trace component of the crude material, and be present in variable quantities in different preparation of the protein fraction.

The work reported here presents a standard system for the use of Fraction 'C' in mice and defines some of the factors which effect the demonstration of the immunosuppressive activity of the material.

MATERIALS AND METHODS

Preparation of fraction 'C'

To 35–40 l of serum were added 1·5 volumes of distilled water and the pH adjusted to 5·0 with glacial acetic acid. After the precipitate formed had settled, the supernatant was pumped from the reservoir on to the top of a 1000-g bed of Whatman DEAE-cellulose equilibrated with 0·03 M acetate buffer pH 5, contained on a 24 cm diameter Buchner funnel. After the serum had all passed through the DEAE, the latter was removed and

suspended as a slurry in 0·2 M pH 5 acetate buffer and poured into two 75 × 5 cm columns. The columns were washed with 0·2 M acetate buffer until protein was no longer eluted. The required fraction, fraction 'C' (Mowbray, 1963a) was then eluted with 0·5 M, pH 5 acetate buffer, and concentrated by ultrafiltration against air in 8/32 in cellophane dialysis tubing.

Preparations were either stored freeze-dried after dialysis against tap water for 24 hours, or stored after adjusting the pH to 7–7·5 with N/10 sodium hydroxide.

Some of the latter preparations were subsequently dialysed against 0·15 M sodium chloride and stored at 4°. All other preparations were stored at −20°. Samples which had been freeze-dried were reconstituted in 0·15 M sodium chloride before use.

Ribonuclease assay

Ribonuclease assay of the fractions was performed in plates of 1 per cent agar, containing 0·1 per cent yeast RNA in 0·1 M acetate buffer pH 5. Samples under test were put in 5-mm wells and incubated overnight at 37°. The diameter of the zone of digested RNA was measured after precipitation of the remaining RNA with 0·25 per cent uranyl acetate in 1·25 per cent trichloracetic acid. Ribonuclease was demonstrated in starch gel electrophoresis by pouring 2 per cent yeast RNA in 0·1 M acetate, pH 5, over the surface of the sliced gel, covering with thin polythene sheet, and incubating at 37° for 16 hours. The gels were stained with amido black dissolved in 40 : 40 : 10 methanol–water–acetic acid, in the usual way. The protein bands stained blue, but the enzyme bands were surrounded by a clear zone in the starch which could be seen by transillumination of the gel. An electrophoresis of a typical preparation is shown in Fig. 1.

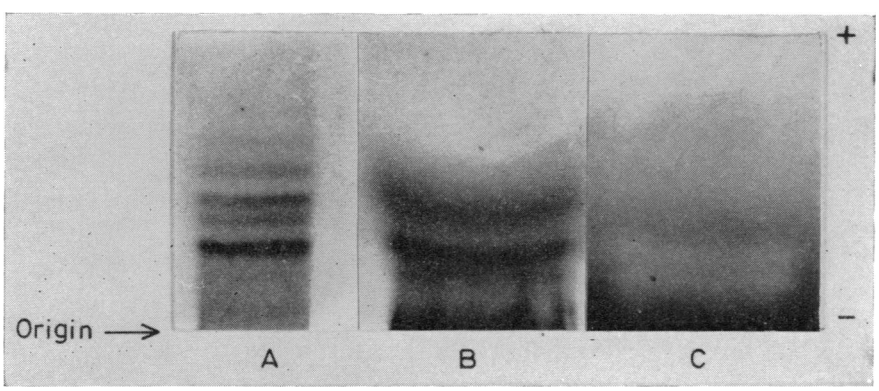

FIG. 1. Starch gel electrophoresis of a typical preparation of Fraction 'C'. A is stained with amido black in the standard way. B has been incubated with RNA and photographed from above. C is the same sample as B photographed by transillumination.

Experimental animals

The effect of intravenous (i.v.) and intraperitoneal (i.p.) injections of Fraction 'C' were studied in C_3H and C57 black mice, and also those of an outbred 'A' strain, all weighing 15–20 g. Since there was no apparent difference in response between these strains, the 'A' strain was used for most of this work.

Haemagglutinin and haemolysin assay

Sheep red cells. All mice were immunized with a single intravenous injection of 0·1 ml of 10 per cent washed sheep red cells. The mice were killed and bled 6 days later.

Serial dilutions of 0·1 ml volumes of serum were made in round-bottomed 5×40 mm polystyrene tubes in barbitone buffered saline pH 7·4. To each tube 0·02 ml of 2 per cent sheep red cells was added and the maximum agglutinating titre recorded after 2 hours incubation at 37°. Guinea-pig complement (0·02 ml) absorbed with sheep red cells and containing 1·5 MHD of complement was added, the tubes were shaken and re-incubated for 4 hours at 37°. The last tube with complete haemolysis was recorded as the haemolysin titre.

Rat red cells. Rat red cells were labelled with ^{51}Cr by the technique of Mollinso and Veall (1955). Mice were injected intravenously with 0·1 ml of labelled rat red cells. Haemagglutinin and haemolysin titres were measured at 6 days as described above, except that 2 per cent rat red cells were substituted for the sheep red cells.

Immune elimination of rat red cells. Blood from the mice injected with labelled rat red cells was obtained daily by retro-orbital puncture. Volumes from 0·05 to 0·2 ml were withdrawn. The samples of blood were lysed in 0·01 per cent ammonia in water. The haemoglobin concentration was determined spectrophotometrically by the absorption at 805 mμ and the radioactivity measured in a well type scintillation counter. The volumes of blood withdrawn were calculated from the haemoglobulin concentration and the radioactivity expressed as counts per minute per millitre of blood. The assumption was made that the haemoglobin concentration in the blood did not alter materially during the course of the experiment.

RESULTS

INTRAPERITONEAL INJECTION

When doses of 1–10 mg of Fraction 'C' were given intraperitoneally in mice before the injection of antigen, there was a normal antibody response to the antigen (Table 1). It was felt that the failure of Fraction 'C' injected by this route to affect antibody production might be due to removal of the Fraction 'C' before it reached the circulation.

TABLE 1

EFFECT OF INTRAPERITONEAL FRACTION 'C' ON THE HAEMAGGLUTININ RESPONSE

Hours between 'C' and sheep cells	No. mice	Geometric mean haemagglutinin titre	S.E.
0	10	1350	224
8	10	480	44
16	9	780	64
24	10	2000	216
48	10	1100	180

INTRAVENOUS INJECTION

With the finding that intraperitoneal injections of Fraction 'C' were ineffective in mice, the effects of intravenous injections were studied. The effect on the immune responses to sheep cells of injection of Fraction 'C' at different times before the administration of the antigen is shown in Fig. 2. In this group of experiments, a standard 4 mg dose of Fraction 'C' was used. The inhibition of the immune response was almost complete when the time interval between injection of Fraction 'C' and injection of the sheep cells was 10–20 hours.

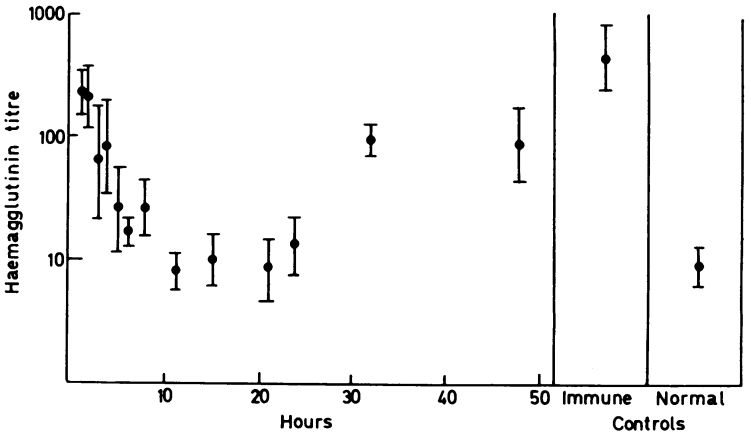

FIG. 2. Effect on haemagglutinin titre of varying the interval between a single 4 mg intravenous injection of Fraction 'C' and the subsequent injection of sheep red cells. (Each point is the geometric mean titre of ten animals; the vertical bar represents ± one S.E.)

HAEMOLYSIN RESPONSE

Similar results were obtained when the haemolysin response was studied. With a 16-hour interval between injection of Fraction 'C' and subsequent injection of sheep cells, suppression of the haemolysin response was complete in twenty-three out of forty animals, and the titre significantly reduced in all but three of the remainder (Table 2).

TABLE 2

EFFECT OF A SINGLE INTRAVENOUS INJECTION OF 4 mg FRACTION 'C' ON THE HAEMOLYSIN RESPONSE TO SHEEP CELLS GIVEN 16 HOURS AFTERWARDS

Treatment	No. mice	Mean haemolysin titre	S.E.
Nil	15	<8	—
Sheep red cells	30	956	108
Sheep red cells+4 mg 'C'	40	<8	—

EFFECT OF DOSAGE

Fig. 3 shows that, with a fixed time interval between injection of Fraction 'C' and the antigen, increasing doses of Fraction 'C' produced progressive diminution in haemagglutinin production in mice, up to a dose of 4 mg per mouse.

IMMUNE ELIMINATION OF RAT RED CELLS

A single intravenous injection of Fraction 'C' did not prevent the immune elimination of ^{51}Cr-labelled rat red cells in mice. The haemagglutinin response at 6 days was unaltered. As rat red cells, unlike sheep red cells, persist in the circulation of normal mice, it was felt that several injections of Fraction 'C' might be needed to affect the immune response to this antigen. Table 3 summarizes the effect of multiple injections and shows that four spaced intravenous injections prevented immune elimination of rat cells, and the haemagglutinin response was barely detectable.

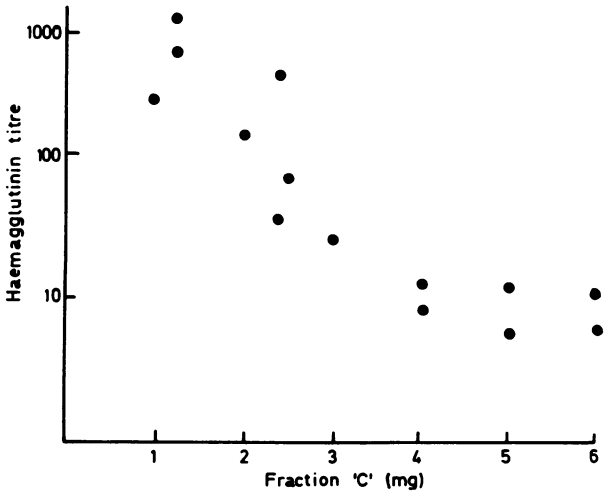

FIG. 3. Effect of dose of Fraction 'C' on the haemagglutinin response to sheep red cells given 16 hours later. (Each point is the geometric mean titre of ten animals.)

TABLE 3

EFFECT OF MULTIPLE INTRAVENOUS INJECTIONS OF FRACTION 'C' ON THE IMMUNE ELIMINATION OF RAT RED CELLS IN MICE, AND THE ANTI-RAT HAEMAGGLUTININ RESPONSE

Interval between 'C' and rat red cells (hours)	Day of immune elimination						Haemagglutinin titre
	1	2	3	4	5	None	
Control	–	8	2	–	–	–	8400
16	–	2	7	1	–	–	620
16, 0	–	–	3	3	–	4	68
16, 0, 24, 48	–	–	–	1	–	9	8

EFFECT OF STORAGE ON ACTIVITY OF DIFFERENT PREPARATIONS OF FRACTION 'C'

Not all batches of Fraction 'C' showed activity when first tested after preparation. During storage either at −20° or at 4°, activity was found to develop during a period of several weeks (Fig. 4). After activity had become maximal, there was no evidence of a fall in activity thereafter for periods of up to 4 months.

A similar effect was noted when freeze-dried batches which had been active before they were freeze-dried were freshly reconstituted. They were then totally inactive. On storage of the reconstituted solutions at either −20° or 4°, the immunosuppressive activity returned (Table 4). This effect was demonstrated with each of four samples of the protein. There was again no evidence of a subsequent fall in activity on storage. The RNase activity increased only by 10–20 per cent during restoration of immunosuppressive activity. Since it appeared possible that some form of reversible aggregation of the material might be occurring during preparation at pH 5 and subsequent storage at pH 7 the effect of leaving the unconcentrated protein solution from the column at pH 7 for 24 hours before concentration was studied. Two batches were each divided into two, one part being adjusted to pH 7 and stored at 4° for 24 hours before concentration; the other

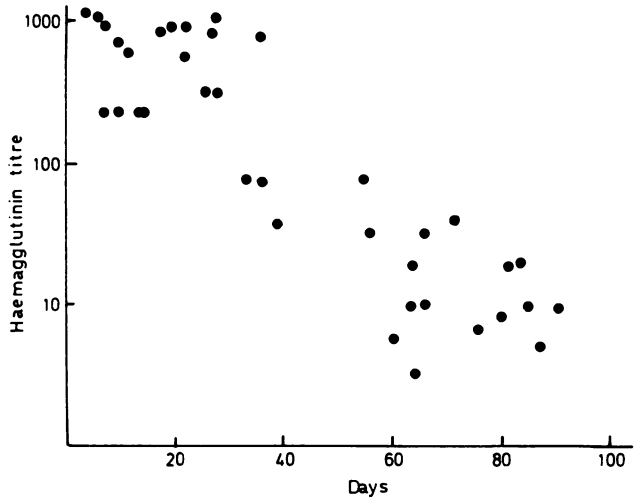

FIG. 4. Effect of length of storage on the immunosuppressive activity of the protein preparation. Data from six preparations of Fraction 'C'. (Each point is the geometric mean titre of five animals.)

adjusted to pH 7 after concentration. Table 5 shows that the first procedure resulted in rapid appearance of activity in both preparations tested, whereas the second did not result in the appearance of full activity for some weeks.

TABLE 4

REAPPEARANCE OF ACTIVITY OF FRACTION 'C' AFTER RECONSTITUTION FROM THE FREEZE-DRIED POWDER

Time after reconstitution (weeks)	Mean haemagglutinin titre			
	Batch 1	Batch 2	Batch 3	Batch 4
0	350	256	380	350
1	95	170	76	500
2	64	45	–	380
4	13·5	32	6·7	11
6	8·2	9·6	6·2	4·6

TABLE 5

DIFFERENCE IN RATE OF APPEARANCE OF IMMUNOSUPPRESSIVE ACTIVITY OF FRACTION 'C' WHEN ADJUSTED TO pH 7 BEFORE AND AFTER CONCENTRATION OF THE PROTEIN

Weeks after preparation	Mean haemagglutinin titre			
	pH 7 before concentration		pH 7 after concentration	
	Batch 1	Batch 2	Batch 1	Batch 2
<1	4·4	8·6	240	100
2	2·6	4·0	240	22
4	3·1	–	135	4·7
6	2·6	–	10	3·3

DISCUSSION

There are probably several reasons for the failure of some workers to demonstrate an immunosuppressive effect with Fraction 'C'. We have shown that the activity of this fraction may be initially absent after freeze-drying, and after chromatography, and it is possible that some batches of material may have been tested for immunosuppression during this phase. This phenomenon may explain the lack of activity of previously active fractions which we have had freeze-dried before sending to other workers for testing, as these were sent before we recognized that the material would need storage after reconstitution.

Davis and Boxer (1965) have confirmed our finding that intraperitoneal injection of the protein is ineffective in mice, although one of us (Mowbray, 1963a) has shown that this route of administration is satisfactory in rats and rabbits.

The enzyme activity of the material was not significantly altered during the period of storage in which the immunosuppressive action was regained. It may be that the active fraction is not the RNase, or that its physiochemical form is altered during storage so that it will only then enter the appropriate cells in the body. In support of the possibility that the enzyme is the active component is the finding that very similar immunosuppressive effects can be obtained with RNase complexed with other proteins (Mowbray and Scholand, 1966). This problem can be resolved only when the RNase from Fraction 'C' is obtained in a pure form.

ACKNOWLEDGMENT

This work was supported in part by a grant to one of us (J.F.M.) from Abbott Laboratories.

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