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# Increase in Macroglobulin Antibodies of Mouse and Pig Following Injection of Bacterial Lipopolysaccharide

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**Summary.** A single intraperitoneal injection of 30 mg. of lipopolysaccharide into a pig gave rise to a rapid evanescent increase in opsonic activity towards *Salmonella typhimurium* C5. This increase was due to antibodies which were shown by density gradient centrifugation and DEAE chromatography to be $\beta_2$M macroglobulins and to be indistinguishable from the 'natural' antibodies present in normal pig serum. The macroglobulin concentration and the opsonic activity were shown to increase for several days but were returning to normal by the 15th day.

## INTRODUCTION

The injection of lipopolysaccharides from Gram-negative bacteria is known to cause profound changes in the immune state of animals for a relatively short time (Rowley, 1956). Many factors vary simultaneously with these changes in susceptibility and attempts to correlate them causally have been made. These include the serum bactericidal activity (Rowley, 1956), serum properdin (Landy and Pillemer, 1956), alkaline and acid phosphatase (Howard, 1959), lysozyme (Hook, Carey and Muschel, 1960), natural antibody levels (Michael, Whitby and Landy, 1962) and the phagocytic capacity of the reticuloendothelial system (Biozzi, Benacerraf and Halpern, 1955; Howard, Rowley and Wardlaw, 1958). Several of these appear to depend on specific antibody for their function, so it is desirable to know whether the increase in antibodies to apparently unrelated antigens which follows lipopolysaccharide injection is due to the release or elaboration of classical 7S $\gamma$-globulin or not. These studies have led to the finding that within 2 days of giving lipopolysaccharide the macroglobulin content of both mouse and pig serum increased remarkably and that the antibody activities were confined to this macroglobulin fraction.

## MATERIALS AND METHODS

### *Animals*

Mice of either sex (weighing 20–25 g.) of an outbred Swiss white strain were used throughout. Two hundred mice were each given an intraperitoneal injection of 50 μg. of a mixture of purified lipopolysaccharides (LP) from 126 different Gram-negative bacteria. Another 200 mice were given the same dose of LP isolated from *S. typhimurium* C5. The pig used was a female 'Large White' weighing 120 lb. and was given 30 mg. of *S. typhimurium* C5 LP intraperitoneally. Both the mice and the pig were sick 24 hours later but 2 days after the LP all animals appeared reasonably well. Mouse blood was obtained from

the retro-orbital venous plexus, pig blood by cardiac puncture. Serum was obtained from these by incubating at 37° for 1 hour, then centrifuging at 3000 rev./min. for 15 minutes.

*Bacterial Lipopolysaccharides*

These had all been prepared by phenol/water extraction (Westphal, Lüderitz and Bister, 1952), the mixed LP was kindly supplied by Dr. O. Westphal and that from *S. typhimurium* was prepared in our laboratory.

*Opsonic Activity of Sera and Fractions*

The methods used for the opsonization of bacteria and the subsequent measurement of the *in vivo* clearance rate of the $^{32}$P-labelled bacteria have been described elsewhere (Jenkin and Rowley, 1961). Recoveries are stated in terms of a biological 'unit', which is that amount of opsonin required to increase the phagocytic index *K* (Biozzi *et al.*, 1955) of unopsonized bacteria by a factor of 0·01. For all the opsonic tests in this paper $^{32}$P-labelled *S. typhimurium* strain C5 was used (Jenkin and Rowley, 1961).

*Mouse Haemagglutinins*

Dilutions of pig sera were titrated against a standard volume of a 1 per cent suspension of mouse red blood cells suspended in saline with 1 per cent normal mouse serum. Results were read by the naked eye after 2 hours at 37° and expressed as reciprocals of the final dilutions showing agglutination.

*Preparation of Antisera*

The preparation of antiserum to opsonized *S. typhimurium* C5 cell walls has been described elsewhere (Turner and Rowley, 1963). Antiserum to pig serum was prepared in rabbits by intravenous injection of 1 ml. of pig serum twice weekly for 3 weeks. Following a 6 weeks rest period the animals received two further injections on days 1 and 5, and were bled 10 days later.

*Protein Analyses*

Total protein concentration of the sera was determined by the Folin-Ciocalteau method and by direct reading in a spectrophotometer at 280 mμ using bovine albumin as a standard.

The major electrophoretic components in each sample were assayed by paper strip electrophoresis in veronal buffer μ 0·075 at pH 8·6 for 16 hours at 6 V./cm., followed by staining with bromphenol blue. The proportions of each component were obtained by reading the strips photometrically in a Spinco 'analytrol'.

*Starch Gel Electrophoresis*

The method and continuous borate buffer system of Smithies (1959) were employed; after 16 hours at 4·2 V./cm. at room temperature the gels were sliced and stained with amido black 10B.

Immuno-electrophoresis was performed at 4° in 1 per cent agar gels in barbital buffer pH 8·4, μ 0·05. Precipitin bands were stained with amido black 10B.

*Macroglobulin Preparation by Sucrose Density Gradients*

Discontinuous sucrose gradients were made in Lusteroid tubes of the SW/39 Spinco rotor by successive layering of 1 ml. amounts of 40, 35, 25 and 10 per cent sucrose in M NaCl. Following 24 hours standing at 4° the gradients were covered with 1 ml. amounts of serum diluted 1 in 3 with saline and centrifuged for 18 hours at 35,000 rev./min. in a Spinco ultracentrifuge as suggested by Kunkel (1960). Each tube was then pierced at its base with a 25 gauge needle and drops taken off slowly into tubes containing 3 ml. of saline. The protein content of each sample was read at 280 mμ in a Shimadzu spectrophotometer.

*Reduction with Mercaptoethanol*

Macroglobulins, isolated by density gradient centrifugation, were degraded by incubating for 2 hours at 37° with 0·1 M mercaptoethanol, pH 7·4.

TABLE 1

CHANGES IN MOUSE SERUM PROTEINS AFTER LIPOPOLYSACCHARIDE

| *Time* | *Albumin (mg./ml.)* | *α + β globulins (mg./ml.)* | *γ-globulin (mg./ml.)* | *Opsonic activity (K units/ml.)* |
|---|---|---|---|---|
| 0 | 24 | 8 | 13 | 0·02 |
| 2 hours | 23 | 9 | 12 | — |
| 6 hours | 25 | 12 | 11·5 | — |
| 24 hours | 27 | 16 | 18 | — |
| 2 days | 28 | 16 | 19 | 0·05 |
| 3 days | 25 | 11 | 16 | — |
| 4 days | 29 | 11 | 17 | — |
| 7 days | 25 | 9 | 16 | — |
| 10 days | 26 | 7·5 | 12 | — |

Each mouse was given 50 μg. of mixed lipopolysaccharides intraperitoneally.
A group of twenty mice was bled at each time interval.

*Removal of γ-Globulin*

Pig serum (2 ml.) was dialysed for 16 hours in the cold against 0·005 M phosphate buffer, pH 6·3, then pushed through a DEAE cellulose column (15 × 1 cm.), equilibrated in the same buffer with 10 lb./in.$^2$ of nitrogen. Adsorbed proteins were eluted with 30 ml. of 0·1 M phosphate, pH 6·3, followed by 30 ml. of 2 M NaCl. Both low and high molarity eluates were concentrated with 'carbowax' to the original serum volume.

## RESULTS

### MICE

Several groups, each of 180 mice, were given 50 μg. of LP intraperitoneally and twenty mice withdrawn at intervals for bleeding. The results of analyses on the sera shown in Table 1 were essentially the same irrespective of the source of the LP. The serum from mice 48 hours after LP had an increased opsonic activity, an increase in α and β proteins, and the macroglobulin content had increased from 4·1 to 5·7 per cent.

PIG

Changes in the major electrophoretic protein groups were not so marked in the pig after LP injection as in the mice. There was, however, a significant rise in the α and β globulins at 2 days which had returned to normal by the 15th day (Fig. 1). This rise was balanced by a corresponding fall in albumin level, and these combined observations were clearly confirmed by starch gel electrophoresis (Fig. 2). A considerable increase in $\alpha_2$ globulin intensity can be seen in the 2, 3 and 5 day samples. Sedimentation at high speed in sucrose gradients was used as a means of quantitating macroglobulin, a typical run is shown in Fig. 3. The method was surprisingly reproducible with a scatter between triplicate assays of around 4 per cent. The changes in macroglobulins by this method are

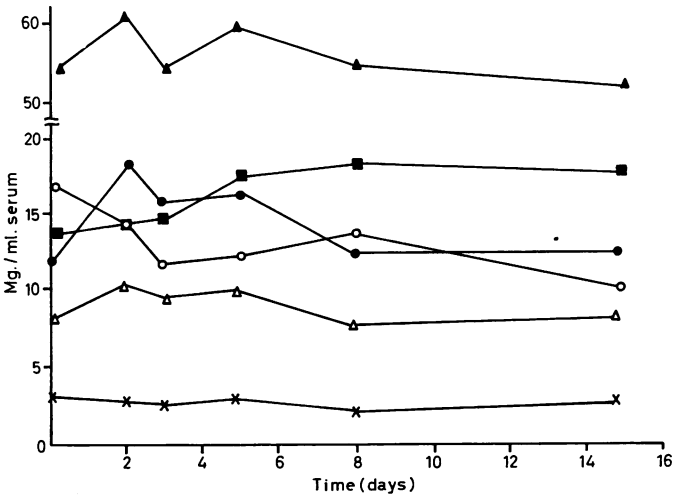


FIG. 1. Changes in pig serum proteins following lipopolysaccharide stimulation as measured by paper electrophoresis. ▲, Total proteins; ○, albumin; ×, $\alpha_1$-globulin: ●, $\alpha_2$-globulin; △, β-globulin; ■, γ-globulin.

shown in Fig. 4 which reveals an increase from 9·8 per cent of total serum protein in the normal pig to 18·6 per cent within 5 days of the LP injection.

On the basis of the sedimentation curves (Fig. 3) the sera were divided into macroglobulins (tubes 1–16) and the remaining proteins. Both fractions were concentrated to the original serum volume by pressure dialysis in L.K.B. ultrafilters.

Each original serum sample was assayed for opsonic activity towards *S. typhimurium* C5 as were several of the macroglobulin fractions, and the non-macroglobulin supernates. Similarly the original sera and the two fractions derived from each of them were tested for haemagglutinating activity against mouse red blood cells. These results are given in Table 2.

The macroglobulins, sedimenting in the sucrose gradient, were further examined by starch gel and immuno-electrophoresis. Identical patterns were obtained with the macroglobulins from all six samples and although both techniques revealed several components, 7S γ-globulins appeared to be absent. A representative pattern by starch gel electrophoresis of the macroglobulins before and after reduction by mercaptoethanol is shown in Fig. 5. The enhanced anodic mobility of all components following treatment with mercaptoethanol suggests a reduction in molecular size and is strong presumptive evidence

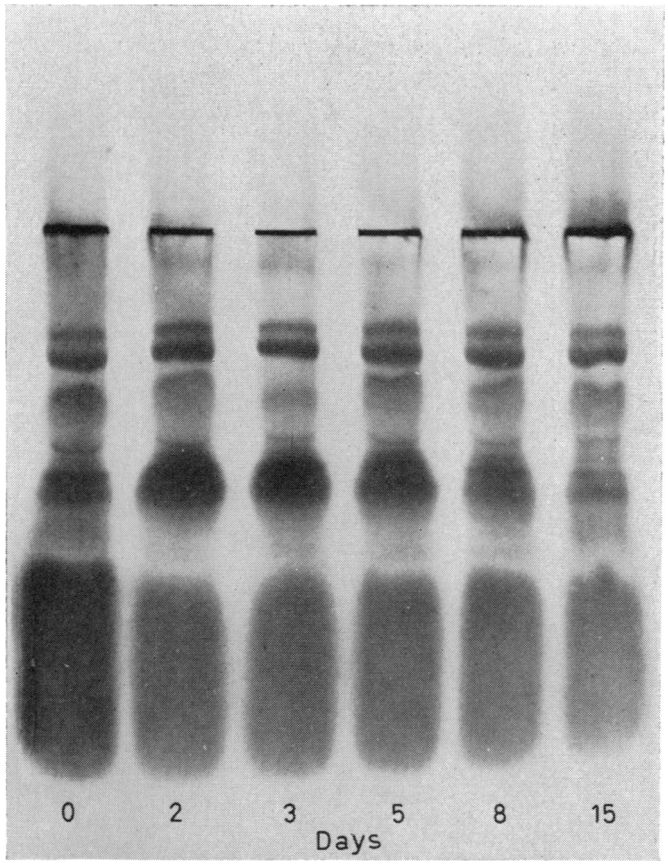


FIG. 2. Starch gel electrophoresis patterns of pig serum following lipopolysaccharide stimulation.

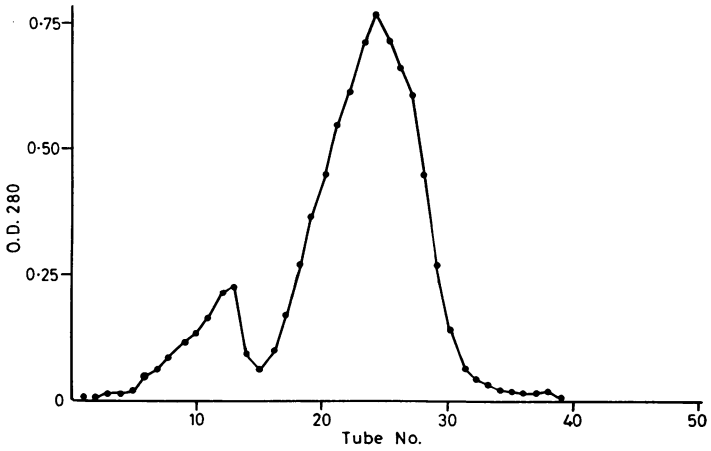


FIG. 3. Sucrose density gradient fractionation of pig serum 3 days after lipopolysaccharide injection.

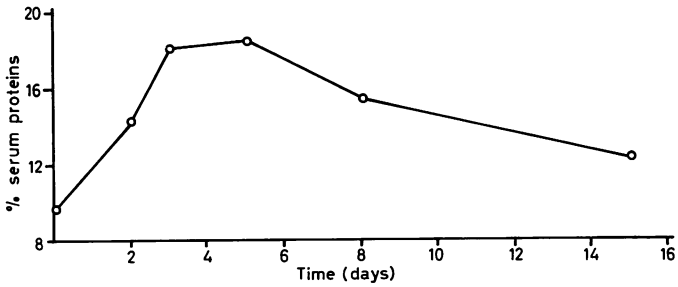


FIG. 4. Changes in macroglobulin content of pig serum, assayed by sucrose density gradient ultracentrifugation, following lipopolysaccharide stimulation.

TABLE 2

ACCUMULATED DATA ON BIOLOGICAL ACTIVITY OF PIG SERA AND FRACTIONS FOLLOWING INJECTION OF 30 mg. *S. typhimurium* C5 LIPOPOLYSACCHARIDE (LP) INTRAPERITONEALLY

| *Days after LP* | *Opsonic activity (K units/ml.)* | | | *Mouse r.b.c. haemagglutinin (reciprocal of final dilution)* | | |
|---|---|---|---|---|---|---|
| | *Serum* | *Macroglobulin* | *Serum without macroglobulin* | *Serum* | *Macroglobulin* | *Serum without macroglobulin* |
| 0 | 40 | 20 | 0 | 800 | 500 | < 50 |
| 2 | 20 | — | — | 800 | 500 | < 30 |
| 3 | 140 | — | — | 1600 | 360 | < 40 |
| 5 | 360 | 360 | 0 | 3200 | 5000 | <120 |
| 8 | 320 | 150 | 0 | 3200 | 1600 | < 50 |
| 15 | 140 | — | — | — | — | — |

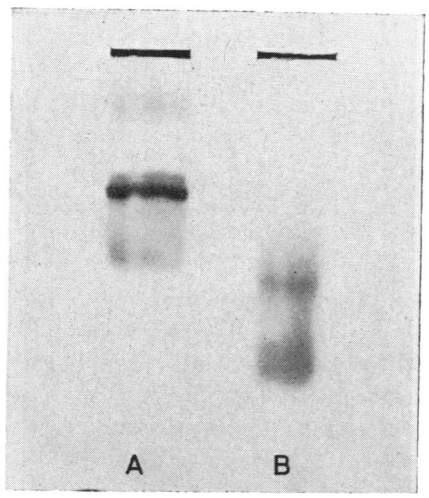


FIG. 5. Starch gel electrophoresis analysis of macroglobulins from pig serum prior to (A) and after (B) reduction with 0·1 M mercaptoethanol.

that this apparently heterogeneous material is all macroglobulin. Immunoelectrophoresis (Fig. 6) showed the presence of both $\beta_2$M and $\alpha_2$M macroglobulins when developed against rabbit anti-pig serum, but only $\beta_2$M bands when developed against rabbit anti-serum to opsonized C5 cell walls (Turner and Rowley, 1963). Paper electrophoresis of the macroglobulin revealed two bands, lying in the $\gamma$-$\beta$ and $\alpha_2$ globulin positions, both of which stained with bromophenol blue and Schiff's acid fuchsin.

Two of the serum samples taken at 5 and 15 days were passed through DEAE cellulose in a manner known to separate $\gamma$-globulins from other more firmly bound proteins devoid of $\gamma$-globulin. When these fractions containing and devoid of $\gamma$-globulin were

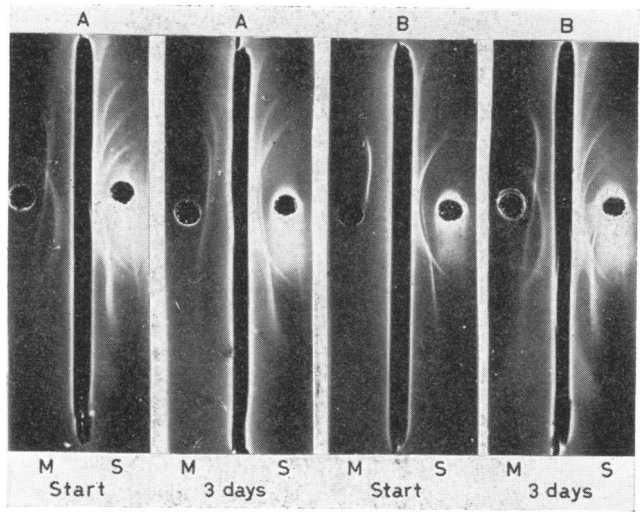


FIG. 6. Immuno-electrophoresis of pig serum (S) and macroglobulins (M) before and 3 days after lipopolysaccharide stimulation. Trough A contained rabbit and anti-pig serum and trough B contained rabbit 'anti-opsonin' serum.

concentrated to the original serum volume, all of the opsonic activity in both the 5 and the 15 day sera was found in the protein material which was eluted at high ionic strength and devoid of 7S $\gamma$-globulin.

## DISCUSSION

In mice surprisingly large variations in serum protein concentrations were induced by injection of the bacterial lipopolysaccharides and these were accompanied by a small increase in the already very low opsonic activity of the serum. It is possible that this elevation of opsonin level was entirely due to the increase in macroglobulin but our techniques were not sufficiently accurate to settle this point with mouse serum, which has such a low opsonic activity for *S. typhimurium*. For this reason we turned to pig serum which is known to be rich in opsonins for this organism (Rowley and Jenkin, 1959), and from which the natural opsonin has recently been purified and shown to occur in the same class of macroglobulins as the natural haemagglutinins (Rowley and Jenkin, 1962; Turner and Rowley, 1963).

The present work with pig serum confirms what was already known with mice (Jenkin and Rowley, 1961) that injection of lipopolysaccharides increases the opsonic activity of the serum for about 8–15 days. This is in keeping with the results of Whitby, Michael, Woods and Landy (1961) who found that LP injections in mice induce transient increases in bactericidal activity of the blood of similar duration and size.

The finding in the present work which we would like to stress is that this increase in opsonic antibody was confined to the macroglobulin fraction which we have previously reported to contain the opsonic activity of normal non-stimulated pigs (Rowley and Jenkin, 1962; Turner and Rowley, 1963). The main evidence for this was that the macroglobulins, isolated by density centrifugation, were devoid of $\gamma$-globulin by immunoelectrophoresis, yet contained all of the opsonic activity. In other words, administration of LP caused a release into the serum of macroglobulin antibody either from pre-existing stores or as a result of new formation. We have no direct evidence to support either of these alternatives. It seems likely, however, that these macroglobulin antibodies as well as many of the enzymes such as acid and alkaline phosphatase are liberated from cells of the R.E.S. (Rowley, 1963) which have been stimulated to undergo rapid division by the uptake of the colloidal LP (Kelly, Brown and Dobson, 1962). The whole change in serum protein distribution cannot, of course, be solely due to increases in macroglobulin, and it seems probable that the various enzymes which have been reported to increase in amount after LP account for much of the change. It has, in fact, been reported that endotoxin uptake causes damage to the lysosomes of cells with consequent liberation of the enzymes contained therein (Janoff, Weissman, Zweifach and Thomas, 1962). Furthermore, the macroglobulin increases themselves cannot solely be due to specific opsonin against *S. typhimurium* and, following from the work of Michael *et al.* (1962), together with our findings with haemagglutinins, one can reasonably postulate that the serum levels of all the natural antibodies being produced at the time of LP injection, were increased perhaps four-fold.

Since the reticulo-endothelial cell proliferation referred to above lasts only a few days (Howard *et al.*, 1958) this, coupled with the limited amount of antibody enhancement and the short half-life of macroglobulins, would account for the restricted duration of the macroglobulin increase. It is also worth noting that this macroglobulin-antibody release is not related to a specific antigen since it can be induced by antigenically unrelated lipopolysaccharides and probably by many colloids (Rowley, 1963).

## ACKNOWLEDGMENTS

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