THE SYNTHESIS AND SECRETION OF IMMUNOGLOBULINS
BY LYMPHOID CELLS IN THE SHEEP
The Primary Response to Salmonella Lipopolysaccharide

By LEONARD S. ENGLISH,* ERIC P. ADAMS, AND BEDE MORRIS
(From the Department of Immunology, John Curtin School of Medical Research, Australian National University, Canberra, Australia)

The levels of immunoglobulin in the blood represent the net result of exchange processes between the intravascular and extravascular compartments of the body and of synthetic and catabolic processes that are proceeding at different rates for different classes of immunoglobulins. Experiments have been done to study antibody synthesis in vitro with mixtures of lymphoid cells and antigen, with cells isolated from lymphoid tissues after antigenic challenge in vivo, and with myeloma cells. In such experiments the physiological mechanisms that regulate the immune response in vivo are absent.

Immune responses can be studied physiologically in single lymph nodes in vivo over many days by collecting and analyzing the constituents of the efferent lymph coming from the nodes (1). We have adapted this experimental approach to study the synthesis and secretion of immunoglobulins during the primary immune response to Salmonella lipopolysaccharide. We have examined the production of both specific and nonspecific immunoglobulins and the temporal sequence in which the different classes of immunoglobulins are synthesized and secreted by cells in two different physiological environments, within the lymph node and in the lymph.

The experiments were designed with the following propositions in mind. If a radiolabeled amino acid is infused continuously via an afferent lymphatic directly into a lymph node it will be taken up from the interstitial fluid by cells within the node and incorporated into newly synthesized protein. Any labeled proteins which are secreted by cells present in the node will leave the node via the efferent lymph, and this protein can be collected quantitatively for analysis. Additionally, experiments can be done in which cells in efferent lymph from the lymph node are collected sequentially after antigenic challenge and these cells incubated with a radiolabeled amino acid in vitro to determine the nature of the protein synthesized and secreted by them.

Materials and Methods

Isolation of Lipopolysaccharide Antigen. Salmonella muenchen organisms which had been maintained on nutrient agar slopes were incubated overnight at 37°C in Hanks' basal salt medium containing 0.5% lactalbumin hydrolysate and 8 g per liter of nutrient broth (Difco Laboratories.

* Present Address: Immunology Department, Memorial University of Newfoundland, Canada.
LEONARD ENGLISH, ERIC ADAMS, AND BEDE MORRIS

Detroit, Mich.). The bacteria were then transferred into a liter of the same medium, incubated for
48 h, washed in 0.9% NaCl solution, and freeze-dried. Lipopolysaccharide (LPS) was extracted
from the bacteria by the phenol method of Westphal et al. (2), and the crude product containing
much bacterial ribonucleic acid was purified by treatment with ribonuclease (3). The LPS was
isolated from solution by centrifugation at 105,000 g for 3 h, redissolved in a small volume of
distilled water, and freeze-dried; 2 mg of this material in 2 ml of sterile 0.9% NaCl solution was
used as an antigen.

Preparation of Antisera

\( \lambda \)-CHAIN ANTISERA. 5 mg of IgG\(_\lambda\) isolated from sheep serum by DEAE cellulose chromatogra-
phy, was dissolved in 1 ml of phosphate-buffered saline (PBS) and mixed with 2 ml of Freund's
complete adjuvant. This mixture was injected intramuscularly into a rabbit three times at
fortnightly intervals. At the end of this period blood was collected and the serum separated and
heated at 56°C for 30 min to remove complement activity. The serum was then absorbed with 2 mg
of sheep IgM/10 ml of serum. The procedure of Fleischman et al. (4) was also used to isolate the
heavy chain component of IgG\(_\lambda\). This component was used for immunization in the same way as
complete IgG\(_\lambda\).

\( \mu \)-CHAIN ANTISERA. The organisms from 20 ml of a concentrated suspension of Salmonella
typhi (Commonwealth Serum Laboratories, Melbourne, Australia) were isolated by centrifuga-
tion, washed, and emulsified with PBS and Freund's complete adjuvant.

A sheep was given two injections of the mixture at an interval of 10 days, and on the 6th day
after the second injection, 100 ml of blood was taken from the jugular vein of the sheep and the
serum separated and inactivated. Salmonella bacteria were suspended in 20 ml of the antisera
and incubated for 1 h at 37°C followed by 2 h at 4°C. The resulting Salmonella-antibody complexes
were spun out, washed six times with 10 ml of PBS, resuspended in 3 ml of 0.9% NaCl solution, and
emulsified with 1 ml of Freund's complete adjuvant. The mixture was injected twice intramuscu-
larly into a rabbit at fortnightly intervals. Serum was collected from the rabbit and absorbed with
2 mg of IgG\(_\lambda\)/10 ml of antisera to give \( \mu \)-chain specificity. The purity of the antisera was
established by double diffusion and immunoelectrophoresis in agar gel.

Analysis of the Immune Response. All experiments were done on conscious Merino ewes, 3-5
yr of age in which the efferent lymphatic from the popliteal lymph node had been cannulated (1).
Antigen was injected under the skin on the lateral aspect of the lower leg, and the efferent lymph
was collected quantitatively in polythene bottles containing a mixture of penicillin, streptomycin,
and heparin. Total cell counts and the number of large cells were determined on samples of lymph
collected during the immune response using a Coulter Counter (Coulter Electronics, Dunstable,
England). At various times before and after antigenic challenge, a plastic catheter was introduced
into an afferent lymphatic duct to enable labeled amino acids to be infused continuously into the
node.

Duplicate 5-ml samples of efferent lymph containing cells that had left the popliteal node were
incubated with \(^{14}\text{C}\)leucine (sp act 311 mCi/mm, Radiochemical Centre, Amersham, U. K.) at a
concentration of 2 \(\mu\text{Ci/ml}\) for 2 h at 37°C while being gently shaken. An excess of \(^{14}\text{C}\)leucine was
added at the end of the incubation and the cells separated from the lymph by centrifugation at 4°C.
The supernate containing the radiolabeled protein secreted by the cells was dialyzed for 2 days at
4°C against either 0.01 M phosphate buffer, pH 8.0 before DEAE cellulose chromatography, or
against 0.01 M phosphate buffer, pH 8.0 containing 0.2 M sodium chloride before Sephadex G-200
chromatography.

To study the production of protein by cells within the popliteal lymph node, \(^{3}\text{H}\)leucine (sp act 1
Ci/mm) was infused into the node via the indwelling afferent lymphatic cannula. The
\(^{3}\text{H}\)leucine was mixed with sterile NaCl solution at a concentration of 30 \(\mu\text{Ci/ml}\) and infused at a
rate of 2 ml/h over periods of 4-8 h. The efferent lymph was collected hourly in polythene
centrifuge tubes containing antibiotics and heparin. An excess of \(^{14}\text{C}\)leucine was added to the
collecting tubes to reduce the synthesis of radiolabeled protein by the cells in the efferent lymph
during the collection period. As soon as the hour's collection was complete, the cells were
separated by centrifugation, washed, and resuspended in isologous lymph which had been col-

1 Abbreviations used in this paper: LPS, lipopolysaccharide; PBS, phosphate-buffered saline.
lected immediately before the infusion. The cells were then incubated at 37°C for 2 h to allow them to secrete the labeled proteins they had synthesized while they were in the lymph node. In some experiments [¹⁴C]leucine was added to the incubation medium in order to compare the production of immunoglobulin by the same cells while they were in the node and subsequently in efferent lymph after they had left the node.

**Separation of Labeled Proteins Secreted by Lymphoid Cells.** 10-ml samples of dialyzed lymph containing 150–300 mg of protein were applied to a column (100 × 3.5 cm) of Sephadex G-200. The proteins were separated by upward displacement using 0.01 M phosphate buffer pH 8.0 in 0.2 M sodium chloride solution. The column was developed at a flow rate of 30 ml/h, and 8.0-ml fractions were collected. The protein content of each fraction was estimated by reading the optical density at 280 nm. Those fractions representing the emerging edge of the first peak (mainly IgM) and the total second peak (IgG) were dialyzed extensively against water and freeze-dried.

**Separation of Labeled Proteins Secreted by Lymphoid Cells.** 2-ml lymph samples containing 30–60 mg protein which had been previously dialyzed against 0.01 M phosphate buffer pH 8.0 were loaded on a column of DEAE cellulose (8 × 2 cm), and the protein fractions were eluted with a phosphate buffer gradient (0.01–0.3 M) (5). The gradient elution was stopped when the optical density of the albumin peak had fallen below 0.20; proteins still absorbed on the column were eluted with 0.3 M phosphate buffer, pH 8.0 in 1 M sodium chloride. Fractions of 3 ml were collected, and portions of each protein peak were pooled. Duplicate samples taken from each peak were assayed for radioactivity as described in the next section; the residues of the pooled samples were dialyzed against distilled water and freeze-dried.

**Estimation of Activity in the Protein Fractions.** Duplicate portions of protein samples obtained either by DEAE cellulose or Sephadex G-200 chromatography were precipitated by adding chilled trichloroacetic acid (TCA) to give a final concentration of 10%. After 2 h at 4°C, the precipitate was isolated by centrifugation, redissolved in 2 ml of 0.25 N sodium hydroxide, and the volume made up to 5 ml with distilled water. The precipitate was finally dissolved in 2 ml of 0.25 N sodium hydroxide. All samples were analyzed for radioactivity after they were oxidized in a Packard 'Tricarb Oxidizer' model 305 (Packard Instrument Co., Inc., Downers Grove, Ill.). The tritium label was collected as H₂O in a dioxane-based scintillation system (Packard Formula 2). The ¹⁴C-label gave ¹⁴CO₂, which was reacted with ethanolamine to produce the carbamate which was dissolved and counted in a methanol/2,5-diphenyloxazole/toluene mixture. The recovery of radioactivity was checked regularly by oxidizing hexadecane standards (Radiochemical Centre, ³H-TRR5 Batch 15, sp act 2.27 μCi/g) and ¹⁴C-CFRS Batch 16, sp act 1.016 μCi/g) and comparing the radioactivity recovered with identical nonoxidized samples dissolved in the same scintillation system. Radioactivity was measured with a Packard Scintillation Spectrometer, model 3002, and the counts converted to disintegrations per minute using both internal standards and quench curves.

**Estimation of the Antibody Activity of the Immunoglobulins.** The antibody activity of the radiolabeled immunoglobulins produced by the lymphoid cells was determined by adsorption onto Salmonella whole organisms. Samples of the immunoglobulin fractions obtained by chromatography were dissolved in 3 ml of PBS, and 0.5-ml portions were used for the binding analyses. Salmonella organisms (10⁹) were suspended in the 0.5-ml samples for 1 h at 37°C and then at 4°C for 2 h. The bacteria and the bound antibody were separated by centrifugation at 19,000 g for 20 min at 4°C and then washed twice with 2-ml vol of PBS. The washed pellet was resuspended in 0.5 ml of PBS, and duplicate samples were assayed for radioactivity after they were combusted in the Tricarb Oxidizer. Equivalent amounts of untreated immunoglobulin were combusted to determine its total radioactivity. The amount of radioactivity removed from the solution by the bacteria was expressed as a percentage to give a measure of the antibody activity of the immunoglobulin sample. Radioactive samples of immunoglobulin prepared from an immune response in sheep to swine influenza virus were used to measure nonspecific adsorption on to the Salmonella organisms.

**Lymph samples collected at intervals throughout the response were assayed for hemagglutinating antibody with LPS-sensitized chicken red blood cells. Twofold serial dilutions of 0.1 ml of lymph were used after removing the complement activity by heating. Mercaptoethanol-resistant antibody was assayed similarly after the samples had been incubated with 0.1 M mercaptoethanol for 30 min.**

**Autoradiography.** Cells incubated in lymph were centrifuged down, washed three times at 4°C
in 10-ml vol of PBS, pH 7.4, and then resuspended in a few drops of the buffer. The cell suspension was then smeared, dried in air, and fixed in methanol-ether (3 vol:1 vol). Pieces of perfused lymph node were fixed in formol-saline, embedded in paraffin, and cut into 5 µm sections. The sections and the cell smears were covered with Kodak AR10 stripping film, and after a suitable time they were developed at 18°C in D19 developer (Eastman Kodak Co., Rochester, N. Y.). The slides were stained with azure A.

Results

The Cellular Response to Salmonella LPS and the Synthesis and Secretion of Radiolabeled Immunoglobulin by the Free-Floating Cells of Lymph In Vitro. The cellular response in the efferent popliteal lymph to a primary injection of LPS is shown in Fig. 1. The response was similar to those that have been described previously for a range of different antigens (1). The maximum output of cells from the popliteal node occurred on the 3rd and 4th day after the injection of antigen and up to 20% of the total cells in the lymph on day 4 were blast cells. By day 7 the output of blast cells had returned to preinjection levels.

The secretion of immunoglobulin by the free-floating cells was examined in vitro throughout the response by incubating the cells in isologous lymph with [3H]leucine. Only very small amounts of labeled protein were secreted by the cells during the first 2 days of the response. The maximum amount of labeled protein was secreted by cells collected on day 4 of the response, and this coincided with the peak of the blast cell response. From day 5 onwards, the secretion of labeled protein fell rapidly so that after day 6, the level of secretion was similar to that before antigenic stimulation. Autoradiographs of the cells incubated with [3H]-labeled leucine showed that it was the blast cells that incorporated most of the radioactivity (Fig. 2 A) although a few small lymphocytes also became heavily labeled.

Analysis of the Secreted Proteins. The labeled proteins secreted by the free-
floating cells were fractionated by DEAE cellulose chromatography and by Sephadex G-200 chromatography. The first protein eluted from the DEAE cellulose column with the phosphate gradient was pure IgG; IgG₁ was the main constituent of the second peak, and most of the albumin was eluted in the third peak. IgM was eluted from the column with 0.3 M phosphate, pH 8.0, containing 1 M sodium chloride. No IgG₁ was found in this final peak. The identity of the peaks was confirmed by immunoelectrophoresis and precipitation of the labeled immunoglobulins with monospecific IgG and IgM antisera. All of the IgG₂, 98% of the IgM, and about 94% of the IgG₁ were precipitated with the specific antisera. It was estimated that over 95% of the protein secreted by these lymphoid cells was immunoglobulin.

The protein secreted by the free-floating cells was also separated by Sephadex G-200 chromatography into IgG and IgM fractions. Cells were collected for these analyses on days 3, 4, and 5 after antigenic challenge, and the results are given in Table I. Although the total amount of immunoglobulin secreted varied considerably in the different experiments, the proportion of IgM to total IgG secreted was relatively constant on each day and for each sheep. On day 3 of the response the proportion of the radioactivity in the IgM fraction comprised nearly 60% of the total radioactivity incorporated into the secreted immunoglobulin. The total amount of radioactivity incorporated into IgM reached a maximum on the 4th day of the response. However, between days 3 and 4 in all experiments there was a large increase in the amount of radioactivity incorporated into the IgG being secreted by the free-floating cells and in consequence, the proportion of radioactivity in the IgM fraction to that in the total immunoglobulin had fallen by day 4.
The Synthesis and Secretion of Protein by Cells Within the Popliteal Lymph Node. The synthesis of protein by cells within the popliteal lymph node was estimated by infusing [3H]leucine directly into the node by way of an afferent lymphatic at various times after LPS was injected into the lower leg. The labeled proteins secreted by the cells within the node entered the lymph which was then collected and analyzed. Each infusion was carried out on a different sheep to avoid extraneous labeled protein, synthesized elsewhere in the body, entering the lymph from the blood stream. In calculating the results it was assumed that all proteins synthesized and secreted by cells within the node enter the efferent lymph exclusively (7).

The distribution of radiolabeled leucine in perfused lymph nodes was examined by autoradiography (Fig. 3 A and B). Labeled cells were restricted to that portion of the lymph node which received lymph from the cannulated afferent lymphatic. After a 3-h perfusion cells in both the cortex and medulla of the perfused segment of the node were labeled. The extent of labeling varied, however, and in general, cells in the peripheral cortex were more heavily labeled than cells in the deep cortex and medulla, although cells adjacent to some of the medullary sinuses were often heavily labeled. In the cortex, the most heavily labeled cells were usually those alongside the pericapsular sinus, and it was assumed that these were near to the point of entry of the perfusate. Cells within germinal centers were not usually labeled.

Less than 10% of the [3H]leucine infused into the node appeared in the efferent lymph either as free amino acid or incorporated into protein. The major part of the leucine must, therefore, have either diffused into the systemic circulation or have been retained in the node. In some experiments lymph from the contralat-
The immunoglobulins secreted by the free-floating cells of lymph were compared in five sheep by determining the percentage incorporation of radioactivity into the three Ig classes when cells were incubated in vitro with [3H]leucine. The number of molecules of each of the immunoglobulin classes produced by the cells is also shown.

The popliteal node was collected simultaneously to measure the extent of synthesis of labeled protein outside the infused node. Over the period of the experiment this was found to be trivial.

The synthesis of immunoglobulin by the cells of the popliteal node was examined in two sheep not given an antigenic challenge and the distribution of [3H]leucine in IgG and IgM determined (Table III). There was no evidence of any cellular response in the lymph coming from the nodes at the time the [3H]leucine was infused, and it was considered that the labeled immunoglobulin was produced by cells within the popliteal node itself.

For the first 2-3 days after the injection of antigen, the level of incorporation of [3H]leucine into immunoglobulin secreted by cells in the node was depressed (Table III). During this time the ratio of IgM to IgG production was at its highest. Cells within the node began to secrete increasing amounts of immunoglobulin into the efferent lymph from the 3rd day of the response, and this coincided with the appearance in the lymph of large numbers of blast cells. By day 4 the output of labeled immunoglobulin from the node had reached a peak. The output of labeled IgM by the cells of the node over days 4 and 5 of the response remained relatively constant in all the sheep examined, whereas the output of labeled IgG increased significantly. Between day 3 and 4 of the response the output of IgM molecules relative to IgG fell from 84/1,000 to 25/1,000. After the peak of the blast cell response in the efferent lymph, cells within the node continued to secrete both classes of immunoglobulins in decreasing amounts for at least as long as 20 days after antigenic challenge. The output of IgM diminished more rapidly than IgG so that 500 h after immunization less than 1% of the total immunoglobulin molecules being secreted by cells in the node was IgM.
Fig. 3. Sections of a popliteal lymph node infused with $[^3H]$leucine via an afferent lymphatic 4 days after primary challenge with LPS. The node was removed 3 h after the infusion was stopped. (A) Diffuse distribution of radioactivity through the cortex of the node. (B) Heavily labeled cells in the medulla of the node. Autoradiograph exposure two wk.
Table III

The Secretion of \(^3\)H-Labeled Immunoglobulins Synthesized by the Cells Within the Popliteal Node During a Primary Response to LPS

| Time after challenge (h) | Percent large cells in lymph | Secretion of \(^3\)H immunoglobulin/h dpm \(\times 10^{-3}\) | Ratio IgM dpm/IgG dpm | Molecules of IgM secreted/1,000 molecules of IgG |
|------------------------|------------------------------|--------------------------------|------------------|---------------------------------|
| Before antigen         | 842                          | 73                            | 0.09             | 15.8                            |
|                        | 503                          | 60                            | 0.11             | 21.3                            |
| 45                     | 5 ± 1                         | 1 ± 1                         | 0.28             | 50.5                            |
| 75                     | 289 ± 20                      | 134 ± 13                      | 0.47             | 84.5                            |
| 87                     | 1,530 ± 53                    | 300 ± 10                      | 0.25             | 35.6                            |
| 94                     | 2,370 ± 2                     | 330 ± 64                      | 0.14             | 25.3                            |
| 174                    | 563                          | 88                            | 0.16             | 28.4                            |
| 510                    | 423 ± 61                      | 24 ± 5                        | 0.96             | 10.0                            |

Mean values are given with their standard deviations for the amount of labeled immunoglobulin secreted hourly for 3 consecutive h during the infusion of \(^3\)H-leucine. The results at each time interval are for separate experiments. The ratio of the incorporation of radioactivity into IgM compared to IgG and the number of molecules of IgM secreted per 1,000 molecules of IgG are also given. The levels of secretion of \(^3\)H immunoglobulin by the cells within the popliteal node before antigenic challenge are shown for two sheep.

Analysis of the immunoglobulins secreted by the cells within the node at various times after challenge showed that IgG\(_1\) was the major component; this was similar to the results obtained with free-floating cells in lymph (Table IV). IgG\(_2\) was produced by cells in the node in greater amounts than IgM. This was in contrast to the results with free-floating cells (cf. Table II).

The Secretion of Immunoglobulin Formed in the Lymph Node by Cells Appearing Subsequently in the Lymph. The cells recovered from the efferent lymph during the infusion of \(^3\)H-leucine into a stimulated node were incubated in vitro to see which radiolabeled immunoglobulins previously synthesized by these cells were secreted by them after they left the node (Fig. 2B). The distribution of radioactivity in the different classes of these ‘preformed’ immunoglobulins was compared to that found in the immunoglobulins synthesized and secreted by the same cells subsequently in the lymph in vitro. A measure of this in vitro synthesis and secretion was obtained by incubating the cells with \(^1^4\)C-leucine. The proportion of immunoglobulins labeled with either \(^3\)H or \(^1^4\)C isotopes enabled a comparison to be made of the synthesis and secretion of immunoglobulins by the same cells in the two different environments, within the node and subsequently in the efferent lymph.

The cells collected each hour during the infusion with \(^3\)H-leucine were separated by centrifugation, and the lymph containing the immunoglobulin secreted by the cells within the node and by the free-floating cells during the collection interval was analyzed for its content of \(^3\)H-labeled IgG\(_1\), IgG\(_2\), and IgM. The cells, which contained \(^3\)H-labeled immunoglobulin synthesized by them while they were in the node, were separated from the lymph by centrifugation and resuspended in an equal volume of lymph which had been collected
The immunoglobulins secreted by the cells within the node were recovered in the efferent lymph during a 3 h infusion of $^{3}$H]leucine into the lymph nodes. The incorporation of radioactivity into each of the immunoglobulin classes is expressed as a percentage of the total Ig secreted. Results at each time interval are for separate experiments.

The Specific Antibody Activity of the Immunoglobulins Produced by the Free-Floating Cells in Lymph and by Cells Within the Popliteal Node. The proportion of specific antibody in each of the labeled immunoglobulin classes was determined using an antigen-binding assay. Representative results obtained at various times in the immune response are shown in Table VI. The proportion of
TABLE V
The Distribution of Radioactivity in the Immunoglobulin Synthesized and Secreted by the Cells Within the Popliteal Node and in the Efferent Lymph at the Height of a Primary Response to LPS

| Sample | Time after antigenic challenge (h) | Percentage distribution of radioactivity within Ig classes |
|--------|-----------------------------------|--------------------------------------------------------|
|        |                                   | IgG₁ | IgG₂ | IgM |
| A      | 96                                | 54.7 | 33.1 | 12.2 |
| B      | 96                                | 46.6 | 14.7 | 38.8 |
| C      | 86                                | 51.7 | 28.4 | 19.9 |
| D      | 96                                | 43.6-55.5 | 9.3-15.5 | 31.0-40.9 |

Sample A: Incorporation of [³H]leucine into immunoglobulin secreted by cells in the node. The result represents the secretion of each Ig class by cells that remained in the node and those that left the node in the lymph during the 1-h infusion period. These migrant cells secreted [³H]labeled immunoglobulin which had been synthesized while they were in the popliteal node.

Sample B: Cells that left the node in the lymph during the infusion of [³H]leucine were incubated with [¹⁴C]leucine in lymph collected before the infusion. The results show the distribution of [¹⁴C] in the immunoglobulin classes secreted during the period of incubation.

Sample C: The secretion of [³H]-labeled immunoglobulin previously synthesized in the node by cells which left the node via the lymph. These cells were present in the node during infusion of [³H]leucine and were incubated in [³H]leucine-free medium for 2 h after they had left the node.

Sample D: The distribution of radioactivity in immunoglobulin secreted by free-floating lymph cells when incubated with [³H]leucine in vitro. The range of values is taken from Table II.

Both IgG₁ and IgM which bound to the antigen increased throughout the response; no antibody activity was detected for IgG₂ until late in the response (about 480 h) when about 9% bound to the antigen. Specific antibody activity in the IgM class was first detected at around 60 h, and on the 3rd and 4th day of the response about 20% of the IgM secreted by either the free-floating cells or by the cells in the node bound to the antigen. This figure was consistent in all the experiments. The activity of the IgM increased later in the response so that by day 7, 40% was bound specifically by antigen. No IgG₁ showed specificity before 79 h; around 20% was specific between days 4 and 7 of the response, and about 40% around day 20. These results were the same for the immunoglobulins produced by both the free-floating cells in lymph and by cells in the popliteal node.

If we assume that the specific radioactivity of the leucine was similar in both IgG and IgM, then an estimate can be made of the relative amounts of antigen-specific IgG and IgM molecules secreted by the free-floating cells. It was calculated that, at the height of the response, about 18 molecules of specific IgG were being produced for every specific IgM molecule, while 20 days after antigenic
challenge there were 73 molecules of specific IgG produced for each molecule of specific IgM. The response to LPS was thus predominantly an IgG response when measured in terms of specific antibody production.

Hemagglutination titers of the efferent lymph indicated that the IgM peak antibody response occurred on days 5 and 6 after challenge (Table VII). Low titers of IgG hemagglutinating activity were first detected on day 5 of the response and reached a peak much later than IgM. At the time that IgG production was at its height as measured by the level of incorporation of $[^3]$H]leucine, IgG showed no hemagglutinating activity at all. IgM was responsible for at least 95% of the specific hemagglutinating activity in the lymph, whereas IgG accounted for most of the specific antibody produced after day 3 when this was measured by the antigen binding assay.

**Discussion**

There are certain deficiencies in the experimental approach we have adopted which prevent quantitative comparisons from being made between the amount
of antibody synthesized by cells within the popliteal lymph node and by the free-floating cells in the efferent lymph when incubated in vitro. Differences in the specific activity of the precursor pool of amino acids in the in vivo experiments and in the in vitro experiments would lead to the synthesis of immunoglobulins of different [3H]leucine content. In addition, the technique of perfusing the popliteal node via an afferent lymphatic duct inevitably led to local differences in the concentration of [3H]leucine in the interstitial fluid of the lymph node. Because of this, cells in the cortical region of the node and in particular those cells adjacent to the point of entry of the afferent lymphatic into the subcapsular sinus were probably presented with an amino acid pool of higher specific activity than elsewhere in the node. If cells secreting different classes of immunoglobulins were located nonrandomly in different areas of the lymph node, this would give rise to immunoglobulin classes of different specific activities. Such conditions did not apply in the in vitro experiments where all the cells were exposed to an amino acid pool of uniform specific activity.

Background Immunoglobulin Synthesis by Cells Within the Popliteal Node. Before antigenic challenge, it was found that the cells of the popliteal node were synthesizing and secreting IgM and IgG. Unlike lymph nodes associated with the gut and mucous surfaces, the popliteal node of the sheep does not normally appear to be in a stimulated state, and it is rare to see blast cells in normal popliteal efferent lymph. It seems, however, that all lymph nodes have a basal level of immunoglobulin synthesis which results in the production of "natural" antibodies and which may in part reflect previous antigenic stimulation. Similar findings have been reported by Roszman et al. (8) who found that the cells of the popliteal lymph nodes of rabbits synthesized immunoglobulins before antigenic challenge. It has also been shown that circulating lymphocytes of humans and splenic lymphocytes of mice secrete immunoglobulins synthesized in vitro in the absence of any specific antigenic challenge (9-11).

After antigenic challenge with LPS, immunoglobulin synthesis in the node was inhibited. This period of inhibition coincided with the recruitment phase of the immune response when there is an increased migration of circulating lymphocytes into the node and when antigen-sensitive cells appear to be selected from the recirculating lymphocyte pool and retained within the node (12).
We assume that the immunoglobulin produced by cells in the node before antigenic challenge would not be specific for the LPS antigen except where cross-reaction occurred. As the products of the antibody-forming cells both in the lymph and the lymph node had a similar specificity for the LPS, it appeared that the inhibition in the basal production of immunoglobulin which followed antigenic challenge continued throughout the response to LPS and was due to some factor or factors produced shortly after antigen entered the lymph node.

The Sequence of Production of Different Classes of Immunoglobulin During the Response to LPS. Although it has been reported that IgM production occurs early in the immune response and is followed sometime later by the production of IgG (13, 14), there are experiments in which a simultaneous production of both Ig classes has been shown to occur (8, 15–17). Several groups of workers have found that only IgM is produced in a primary response to endotoxin (13, 18–20), while others have claimed that although the response is predominantly IgM there is a late production of IgG (21–23). In these reports antibody activity was assayed by passive hemagglutination or by the enumeration of plaque-forming cells and was attributed to IgM. When antibody production was examined by either precipitin or antigen-binding techniques, both IgM and IgG were identified early in responses to endotoxin (24, 25).

In the present experiments, while both IgM and IgG were detected initially on day 3, only IgM had measurable antigen-binding activity at this time. On day 4 of the response, when there were 18 times more antigen-specific IgG molecules than IgM, as measured by the binding assay, the hemagglutination assay only detected specific IgM. It was not until later in the response that the IgG fraction had hemagglutinating activity. As antigen-binding assays and hemagglutination assays appear to be biased towards the identification of one or other of the immunoglobulin classes and as the affinities of the immunoglobulins are continually changing during a response, descriptions of the sequence of immunoglobulin synthesis will depend on the particular assay system used and on the time at which the response is analyzed.

The Cells Involved in Immunoglobulin Synthesis in the Lymph and in the Lymph Node. A variety of phenotypes are represented among cells that synthesize antibody (26, 27). The predominant cell type in lymph which synthesizes and secretes antibody is a blast cell; in the lymph node as well as the blast cells, classical plasma cells produce antibody. Hay (28) made an estimate of the contribution made by the blast cells in lymph to the total hemagglutinating antibody produced in a primary response to LPS in sheep and concluded that these cells were responsible for the synthesis of at least half of the total IgM antibody. Further evidence of the capital role played by the blast cells in the immune response comes from the present experiments. When cells from efferent lymph were incubated in vitro with [3H]leucine, it was the blasts that incorporated most of the radioactive label. In addition the secretion of immunoglobulin by the cells within the node reached a peak coincident with the peak of the blast cell response in the lymph, suggesting further that these cells were producing most of the antibody in the node at this time. Most of the secretion of IgM occurred between the 3rd and 6th day of the response when blast cells were most obvious in both the lymph and the lymph node.
The pattern of secretion of immunoglobulins by cells within the node and by free-floating cells in the efferent lymph was different. The cells in the lymph, when incubated in vitro, secreted a higher proportion of IgM than did the cells in the node. This observation could be explained either by a difference in the relative proportions of IgM- and IgG-secreting cells in the lymph and in the node or by a relative suppression of IgM production or a relative enhancement of IgG synthesis and secretion within the node. The idea that IgM production was inhibited relative to IgG in the environment of the lymph node is supported by the observation that the same population of cells synthesized a significantly higher proportion of IgM when incubated in lymph in vitro than when they were in the node. This was not due to further differentiation of the cells after they had left the node, for the results showed that as mature plasma cells accumulated in the node and the blast cells disappeared from the lymph, IgG synthesis increased relative to IgM synthesis. We concluded that mature plasma cells produced largely IgG, whereas blast cells in the lymph and in the node synthesized both IgG and IgM.

The Synthesis of Nonspecific Antibody. Antibodies produced in an immune response are known to have a wide range of affinities for the antigen that evoked their production, and this makes it difficult to decide just what constitutes nonspecific antibody. There are many reports that the majority of immunoglobulins produced in an immune response appears to have no specificity for the antigen (8, 29-31). Quite apart from acting as a specific inductive stimulus for antigen-sensitive cells, bacterial LPS have been shown to stimulate bone marrow-derived lymphocytes to divide (32), to have potent adjuvant effects on antibody formation (33), and to act as a substitute for thymus-derived cells in stimulating immune responses to various antigens (34-35). Coutinho and Möller (36) have also reported that LPS not only stimulates bone marrow cells to divide in vitro, but also induces them to produce a variety of antibodies. The present findings in sheep provide evidence that in vivo, LPS evokes the production of a large proportion of immunoglobulin that does not appear to have any affinity for the antigen at least in so far as the antigen-binding and hemagglutinating assays can determine.

The dose of LPS and the route of challenge used to induce the immune response is probably important in determining the relative proportions of specific and nonspecific immunoglobulin produced (19, 37, 38). We have done experiments in sheep with low doses of LPS which have shown that the percentage of nonspecific IgG and IgM was reduced significantly under these circumstances. However, very little is known concerning the dose-response relationships which affect the production of specific and nonspecific antibody.

There may be limitations in the antigen-binding assay which would allow low affinity antibody, bound initially to the antigen, to be lost during the washing procedures and thus lead to its classification as nonspecific. Robbins et al. (25) found that higher concentrations of Salmonella organisms were required to adsorb out IgM antibody from serum than were necessary to remove the IgG. This result suggested that IgM antibody had a lower affinity for antigen than IgG. It may be that nonspecific immunoglobulin against LPS is directed against some antigenic determinants on the LPS molecule not tested for in the binding
assay. If these determinants were not suitably exposed on the surface of the whole organism or were sterically hindered in such a way that the antibody was prevented from interacting with them, this antibody would be classified as nonspecific. It has also been shown that antibody can be produced against the lipid A moiety of the LPS molecule (39). The nonspecific immunity that can be induced to serologically unrelated strains of bacteria by the injection of another strain of bacteria, LPS or lipid A (40), can be explained by the production of antibodies against lipid A, since this is common to all the serologically unrelated bacteria to which cross-immunity has been generated (41). The binding assay used in the present experiments would be unlikely to detect lipid A antibodies, as these determinants are not exposed on the surface of the Salmonella organisms (42).

Summary

The primary response of the popliteal node to Salmonella lipopolysaccharide was studied in the sheep. All three classes of immunoglobulin IgG₁, IgG₂, and IgM were produced by both free-floating cells in the lymph and by cells within the lymph node throughout the immune response which extended over a period of at least 20 days. Most of the immunoglobulins were found to be nonspecific for the antigen when tested by a binding assay. It was calculated from the binding assay that far more antigen-specific IgG molecules were produced than IgM molecules. The proportion of IgM and IgG₂, which showed affinity for Salmonella organisms increased throughout the response. IgG₃ had no affinity for the antigen until around 480 h after challenge. When a hemagglutination assay was used to measure antibody production, most of the specific antibody produced during the response was found to be IgM.

Blast cells produced most of the immunoglobulin during the first 4 days of the response, and these cells were responsible for almost all of the IgM production.

Differences were observed in the relative amounts of IgG and IgM produced by the cells within the node and by free-floating cells in the efferent lymph. The free-floating cells in lymph synthesized and secreted relatively more IgM and relatively less IgG than did cells within the lymph node. Both populations of cells, however, secreted much more IgG than IgM.

References

1. Hall, J. G., and B. Morris. 1963. The lymph-borne cells of the immune response. Q. J. Exp. Physiol. Cogn. Med. Sci. 48:235.
2. Westphal, O., O. Lüderitz, and F. Bister. 1952. Über die Extraktion von Bacterien mit Phenol/Wasser. Z. Naturforsch. Teil. B. Anorg. Chem. Org. Chem. Biochem. Biophys. Biol. 7B:148.
3. Fensom, A. H., and G. W. Gray. 1969. The chemical composition of the lipopolysaccharide of Pseudomonas aeruginosa. Biochem. J. 114:185.
4. Fleischman, J. B., R. H. Pain, and R. R. Porter. 1962. Reduction of γ-globulins. Arch. Biochem. Biophys. Acta 1(Suppl.):174.
5. Fahey, J. L., P. F. McCoy, and M. Goulian. 1958. Chromatography of serum proteins in normal and pathologic sera: the distribution of protein-bound carbohydrate and
cholesterol, siderophilin, thyroxin-binding protein, B12-binding protein, alkaline, and acid phosphatases, radio-iodinated albumin, and myeloma proteins. *J. Clin. Invest.* 37:272.

6. Heimburger, N., K. Heide, H. Haupt, and H. E. Schultze. 1964. Bausteinanalysen von Humanserumproteinen. *Clin. Chim. Acta.* 10:293.

7. Hall, J. G., B. Morris, G. Moreno, and M. C. Bessis. 1967. The ultrastructure and function of the cells in lymph following antigenic stimulation. *J. Exp. Med.* 125:91.

8. Roszman, T. L., J. D. Folds, and A. B. Stavitsky. 1971. A kinetic and quantitative analysis of antibody and protein synthesis during the primary response to keyhole limpet haemocyanin. *Immunology.* 20:1041.

9. Van Furth, R., H. R. E. Schuit, and W. H. Schultze. 1966. The formation of immunoglobulins by human tissues in vitro. IV. Circulating lymphocytes in normal and pathological conditions. *Immunology.* 11:29.

10. Chessin, L. N., P. R. Glade, R. Asofsky, P. D. Baker, R. Reisfeld, and W. Terry. 1968. Studies on human peripheral blood lymphocytes in vitro. V. Biosynthesis of immunoglobulins. *J. Immunol.* 101:458.

11. Vitetta, E. S., and J. W. Uhr. 1972. Cell surface immunoglobulin. V. Release from murine splenic lymphocytes. *J. Exp. Med.* 136:876.

12. Hay, J. B., and B. Morris. 1976. The generation and selection of specific reactive cells by antigen. *Br. Med. Bull.* 32:125.

13. Bauer, D. C., and A. B. Stavitsky. 1961. On the different molecular forms of antibody synthesized by rabbits during the early response to a single injection of protein and cellular antigens. *Proc. Natl. Acad. Sci. U. S. A.* 47:1867.

14. Benedict, A. A., R. J. Brown, and R. Ayengar. 1962. Physical properties of antibody to bovine serum albumin as demonstrated by hemagglutination. *J. Exp. Med.* 115:195.

15. Freeman, M. J., and A. B. Stavitsky. 1965. Radioimmuno-electrophoretic study of rabbit anti-protein antibodies during the primary response. *J. Immunol.* 95:981.

16. Osler, A. G., J. J. Mulligan, Jr., and E. Rodriguez. 1966. Weight estimates of rabbit anti-human serum albumin based on antigen-binding and precipitin analyses: specific hemagglutinating activities of 7S and 19S components. *J. Immunol.* 96:334.

17. Plotkin, D. H., S. Konttinen, A. B. Stavitsky, and O. Mäkelä. 1968. Characterisation of the early primary antibody response to the 4-hydroxy-3-iodo-5-nitrophenylacetate (NIP) and the 2,4-dinitrophenyl (DNP) determinants. *Immunology.* 15:799.

18. Bauer, D. C., M. J. Mathies, and A. B. Stavitsky. 1963. Sequences of synthesis of γ-1 macroglobulin and γ-2 globulin antibodies during primary and secondary responses to proteins, Salmonella antigens, and phage. *J. Exp. Med.* 117:889.

19. Britton, S., and G. Möller. 1968. Regulation of antibody synthesis against *Escherichia coli* endotoxin. I. Suppressive effect of endogenously produced and passively transferred antibodies. *J. Immunol.* 100:1326.

20. Friedman, H. 1973. Cellular and molecular aspects of the response to a bacterial somatic antigen. *J. Infect. Dis.* 128:561.

21. Landy, M., R. P. Sanderson, and A. L. Jackson. 1965. Humoral and cellular aspects of the immune response to the somatic antigen of Salmonella enteritidis. *J. Exp. Med.* 122:483.

22. Holmgren, J. 1970. The antibody response in rabbits to *E. coli* O antigen. *Int. Arch. Allergy Appl. Immunol.* 37:546.

23. Ahlstedt, S., J. Holmgren, and L. A. Hanson. 1973. The primary and secondary antibody response to *Escherichia coli* 06 lipopolysaccharide analyzed at the humoral and cellular level. Amount and avidity of the antibodies in relation to protective capacity. *Immunology.* 24:191.
24. Jonas, W. E. 1969. Immunoelectrophoretic analysis of sheep serum using guinea-pig antiserum to particulate antigens treated with sheep antiserum. *Res. Vet. Sci.* 10:397.
25. Robbins, J. B., K. Kenny, and E. Suter. 1965. The isolation and biological activities of rabbit γM and γG anti-Salmonella typhimurium antibodies. *J. Exp. Med.* 122:385.
26. Cunningham, A. J., J. B. Smith, and E. H. Mercer. 1966. Antibody formation by single cells from lymph nodes and efferent lymph of sheep. *J. Exp. Med.* 124:701.
27. Hay, J. B., M. J. Murphy, Jr., B. Morris, and M. C. Bessis. 1972. Quantitative studies on the proliferation and differentiation of antibody-forming cells in lymph. *Am. J. Pathol.* 66:1.
28. Hay, J. B. 1970. The role of fixed and migratory cells in immunological reactions. Ph.D. Thesis. Australian National University, Canberra.
29. Urbain-Vansanten, G. 1970. Concomitant synthesis in separate cells of non-reactive immunoglobulins and specific antibodies after immunization with tobacco mosaic virus. *Immunology.* 19:783.
30. Bosman, C., and J. D. Feldman. 1970. The proportion and structure of cells forming antibody, γG and γM immunoglobulins, and γG and γM antibodies. *Cell. Immunol.* 1:31.
31. de Vos-Cloetens, C., V. Minsart-Baleriaux, and G. Urbain-Vansanten. 1971. Possible relationships between antibodies and non-specific immunoglobulins simultaneously induced after antigenic stimulation. *Immunology.* 20:955.
32. Andersson, J., G. Möller, and O. Sjoberg. 1972. Selective induction of DNA synthesis in T and B lymphocytes. *Cell. Immunol.* 4:381.
33. Neter, E. 1969. Endotoxins and the immune response. *Curr. Top. Microbiol. Immunol.* 47:82.
34. Watson, J., R. Epstein, I. Nakoinz, and P. Ralph. 1973. The role of humoral factors in the initiation of in vitro primary immune responses. II. Effects of lymphocyte mitogens. *J. Immunol.* 110:43.
35. Jones, J. M., and P. D. Kind. 1972. Enhancing effect of bacterial endotoxins on bone marrow cells in the immune response to SRBC. *J. Immunol.* 108:1453.
36. Coutinho, A., and G. Möller. 1973. B-cell mitogenic properties of thymus-independent antigens. *Nat. New Biol.* 245:12.
37. Weidanz, W. P., A. L. Jackson, and M. Landy. 1964. Some aspects of the antibody response of rabbits to immunization with enterobacterial somatic antigens. *Proc. Soc. Exp. Biol. Med.* 116:832.
38. Svehag, S. E., and B. Mandel. 1964. The formation and properties of poliovirus neutralizing antibody. I. 19S and 7S antibody formation: differences in kinetics and antigen dose requirement for induction. *J. Exp. Med.* 119:1.
39. Lüderitz, O., C. Galanos, V. Lehmann, M. Nurminen, E. T. Reitschel, G. Rosenfelder, M. Simon, and O. Westphal. 1973. Lipid A: chemical structure and biological activity. *J. Infect. Dis.* 128(Suppl.):17.
40. Howard, J. G., D. Rowley, and A. C. Wardlaw. 1958. Investigations on the mechanism of stimulation of non-specific immunity by bacterial lipopolysaccharides. *Immunology.* 1:181.
41. Lüderitz, O., K. Jann, and R. Wheat. 1968. Somatic and capsular antigens of gram-negative bacteria. In Comprehensive Biochemistry. Florkin, M., and E. H. Stotz, editors. American Elsevier Publishing Co., Inc., New York. 105.
42. Woer, W., and P. Alaupovic. 1971. Studies on the protein moiety of endotoxin from gram-negative bacteria. Characterization of the protein moiety isolated by phenol treatment of endotoxin from *Serratia marcescens* 08 and *Escherichia coli* 0 141:K85 (B). *Eur. J. Biochem.* 19:340.