Antibody Response to Bacterial Antigens: Characteristics of Antibody Response to Somatic Antigens of Salmonella typhimurium

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The character of the antibody response in the rabbit to Salmonella typhimurium somatic (O) antigen was similar to the response to each of several serotypes of Shigella flexneri O antigens, namely a predominance of production of immunoglobulin M (IgM) antibody. Lipopolysaccharide protein (LPSP) and lipopolysaccharide (LPS) fractions of Salmonella O antigen differed significantly in both quantitative and qualitative aspects of their immunogenicity. LPSP elicited high levels of agglutinins and also induced the production of a significant amount of immunoglobulin G (IgG) antibody at a late period. LPS antigen elicited low levels of agglutinins which were exclusively IgM antibody. These results suggested that the chemical nature of the antigen is one important factor in the determination of the character of the antibody response. Further, it is suggested that the protein moiety of the O antigen complex is a carrier active in allowing induction of early IgM and of late IgG antibodies; in contrast, the lipid moiety may compete with this action of the carrier protein, thereby suppressing IgG antibody in the primary stage of the antibody-forming process.

A variety of factors influence the types of antibodies produced in response to various antigens. Uhr (22) reviewed these differing responses, which depend on the quantity and the nature of the antigen in addition to other factors, and indicated the difficulty in generalizations concerning antibody formation.

Our previous studies on the immune responses of rabbits to Shigella flexneri (7, 21) and Candida albicans (6) demonstrated the presence of both immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies in hyperimmune sera. The time of appearance of the two types of antibodies during the course of immunization with these two microbial antigens differed. Agglutinins of S. flexneri were found exclusively in the IgM fraction in the early period of immunization. In contrast, in response to C. albicans considerable amounts of early IgG antibody were found in addition to early IgM (6). It was further shown that the ratio of IgG to IgM antibodies for C. albicans was remarkably higher than that for S. flexneri in hyperimmune serum (6, 7). These results suggested that the nature of the cell wall constituents is an important factor in determining the class of antibodies induced.

The present study compares the antibody response of the rabbit to Salmonella somatic antigen with the responses to Candida or Shigella somatic antigens previously reported by the use of identical immunizing methods. A further comparison was made of immune responses to two classes of extracted soluble-antigen complexes, lipopolysaccharide protein (LPSP) and lipopolysaccharide (LPS), administered by subcutaneous injection. Finally, the specificity of the two classes of antibodies for Salmonella O antigen 1 was examined by using bacteria lysogenized with P22 phage (20, 26, 27).

MATERIALS AND METHODS

Strains used. Salmonella typhimurium LT-2 and a derivative lysogenized with P22 phage were used as antigens for the immunization of rabbits and for agglutination tests.

Preparation of immune sera. New Zealand albino rabbits, male, weighing 3.5 kg were injected intravenously five times at 3-day intervals with bacterial antigens prepared as previously described (7). Immune sera for each crude, extracted antigen (described below) were prepared as follows; 5 mg of LPS saccharide antigen or 0.5 mg of LPSP antigen was dissolved in 2 ml of distilled water and then mixed with an equal volume of Freund's complete adjuvant. A rabbit received an injection in the foot pad of one
of the two antigen preparations and was bled from its
ear veins once each week after injection.

Fractionation of immune sera. Rabbit immune sera
were fractionated by gel filtration on a Sephadex
G-200 column (Pharmacia, Uppsala, Sweden) as
described previously (7). The antibody fractions were
mixed with 0.15 M 2-ME at 37°C for 3 hr (12) and
then were immediately titrated by agglutination.

Preparation of crude antigens. Crude LPSP complex
of S. typhimurium was prepared according to a cold
trichloroacetic acid extraction method (19). Crude
LPS was prepared according to a phenol-water
method (24).

Characterization of antigen extracts. Each crude
eat was fractionated by gel filtration through a column
(2.5 by 45 cm) of Sephadex G-200. The gel
was equilibrated with 0.067 M phosphate buffer (pH
7.6), and 2 ml of 2% crude extract in the same buffer
was applied. The total sugar content of each fraction
was assayed at 560 nm with Molisch reagent (10).
Nucleic acid and protein contents were estimated by
optical densities at 260 and 280 nm, respectively.

Removal of nucleic acid. Nucleic acid in the first
main fraction of the crude LPS on Sephadex G-200
column was removed by precipitation with cetyltri-
methylammonium bromide (Cetavlon) by the method
of Westphal and Jann (24).

Detection of monofactor for somatic antigen 1 from
antibody fractions. An adsorption test was carried out
by a method previously described (26), with slight
modification. Immune serum to S. typhimurium lyso-
genized with phage P22 and its IgM and IgG fractions
were adjusted to yield an agglutinin titer of 1:160
dilution against homologous antigen. The immune
serum and antibody fractions were first adsorbed with
heated cells of a nonlysogenic strain of S. typ-
himurium and were then tested for ability to agglutinate
heated cells of lysogenic and nonlysogenic strains.

RESULTS

Antibody response in rabbits after intravenous
injections with S. typhimurium. The production of
IgM and IgG antibodies with agglutinin activities
was studied in connection with the time of immu-

nization. Two rabbits were inoculated intra-
venously five times at 3-day intervals with heated
cells of S. typhimurium (Fig. 1). Agglutinins ap-
ppeared in their sera 4 days after the first injection
and reached a peak on the 12th day; they then
decayed slowly after the 16th day. Antiserum ob-
tained at each period was treated with 2-ME and
titered by agglutination. The 2-ME-resistant ag-
glutinins increased rapidly after the third injec-
tion. The approximate proportions of IgM and
IgG antibodies in the immune sera were deter-
mained at several intervals by measuring the ag-
glutinin activities of the first and the second
fractions after gel filtration of the immune sera on
Sephadex G-200 and by tests for 2-ME sensitivity.
The IgM antibody appeared at an early period
during immunization and was the predominant
antibody through the 23rd day. The IgG antibody
was produced slowly, but it approached the level
of IgM antibody in activity by the 23rd day. The
results indicate that the character of the antibody
response to S. typhimurium O antigen is quite
similar to the response to Shigella flexneri O
antigen (7), demonstrating a predominant IgM
production.

Antibody response to LPSP antigen of Sal-
onella typhimurium. The soluble somatic antigen
extracted by cold trichloroacetic acid yielded one
narrow peak active with Molisch reagent upon
Sephadex G-200 filtration. This peak and the
entire eluate did not contain a significant amount
of substances absorbing at 260 and 280 nm
(Fig. 2).

The peak fraction was pooled and used for
immunization of rabbits. Since extracted antigens
were toxic to rabbits when injected intravenously,
subcutaneous injections with Freund's complete
adjuvant were performed with extracted antigens.
Agglutinins against LPSP emerged within 1 week
after a single injection of 0.5 mg of antigen and
reached a peak on the 2nd week; they then per-
sisted for several weeks (Fig. 3). Immune sera
obtained at each period were treated with 2-ME
and titered by agglutination. The 2-ME-resistant
antibody appeared rapidly about the 2nd week
after the injection and increased gradually. Rela-
Antibody response to Salmonella O antigen

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Fig. 2. Gel filtration of crude LPS antigen of S. typhimurium through Sephadex G-200. The crude antigen solution was applied to a column of Sephadex G-200 equilibrated with 0.067 M phosphate buffer (pH 7.6) containing 0.2 M NaCl and was eluted with the same buffer. Polysaccharide was assayed at an optical density of 560 nm with Molisch reagent.

Fig. 3. Antibody production in the rabbit after a single injection of LPS antigen. The rabbit was given an injection of 0.5 mg of antigen with Freund's complete adjuvant. Immune sera obtained at each period were assayed for antibody activity by the tube agglutination method before and after treatment with 2-ME. White bars show relative proportions of IgM antibody activity; shaded bars, IgG antibody activity.

Fig. 4. Gel filtration of crude LPS antigen of S. typhimurium through Sephadex G-200 column. Crude antigen solution was applied to a column of Sephadex G-200 equilibrated with 0.067 M phosphate buffer (pH 7.6) containing 0.2 M NaCl and was eluted with the same buffer. Polysaccharide was assayed at optical density of 560 nm with Molisch reagent.

utive proportions of the two types of antibodies produced at each period of immunization were estimated after gel filtration through Sephadex G-200 for their antibody activities in relation to 2-ME sensitivities. IgG antibodies predominated in antisera after the 3rd week (Fig. 3). These results indicate that soluble complete O antigen (LPS) induces a high IgG antibody response in contrast with the intact cell antigen.

Antibody response to LPS antigen of S. typhimurium. The crude LPS fraction extracted by phenol-water, when passed through Sephadex G-200, yielded a broad peak active with Molisch reagent (Fig. 4). This peak contained a considerable amount of substances absorbing at 260 and 280 nm. These substances could be considered to be cytoplasmic components of nucleic acid or nucleoprotein; they were removed from LPS fraction after treatment with Cetavlon. After removal of ribonucleic acid (RNA) by Cetavlon, 5 mg of antigen per rabbit was used for immunization because 0.5 mg of LPS antigen did not elicit appreciable antibody in a preliminary experiment. Two rabbits each were given a sub-
cutaneous injection of antigen mixed with Freund's complete adjuvant. The agglutinin in the serum of one rabbit (R-13) emerged within 1 week after the single injection and reached a peak on the 4th week. Another rabbit (R-14) which did not produce appreciable antibody for 2 weeks after the first injection was given a second injection of 5 mg of antigen. In response to the booster inoculation, agglutinins appeared in the serum during the 3rd week and reached a peak during the 4th week (Fig. 5). Immune serum obtained at weekly intervals after immunization of the rabbit (R-13) with a single injection contained agglutinins which were completely 2-ME sensitive. Similarly, immune serum from another rabbit (R-14), given a booster injection at the 2nd week, contained agglutinins which were totally 2-ME sensitive. These results indicate that the immunogenicity of LPS is comparatively weak and is capable of inducing only IgM antibody.

**Antibody response to S. typhimurium somatic O antigen as a function of antigenic stimulation.** To examine the influence of the intensity of immunization on the antibody response, four groups of rabbits (consisting of two rabbits per group) were given one to four intravenous injections of heated cells of *S. typhimurium* containing O antigen (Fig. 6). In the rabbits of the first and the second groups, which were injected once or twice with antigen, agglutinins appeared in the sera on the 8th day and then declined. Antibody activity was found only in the IgM fraction of antisera either on the 8th day or on the 16th day. In the response of rabbits of the third group to three inoculations of antigen, a small amount of IgG antibody appeared in the sera on the 16th day. Finally, rabbits of the fourth group were given four injections of antigen. The 2-ME-resistant antibody increased in amount, and significant amounts of IgG antibody also appeared in the sera on the 16th day. Thereafter, all the antibody activities declined. These results indicate that intensive immunization is a prerequisite for induction of detectable IgG antibody during immunization with *S. typhimurium* O antigen.

**Specificity of the two types of antibodies appearing late in immunization.** Monofactorial activity of the two types of antibodies was examined by detecting antibody factor for one O antigen, antigen 1. Immune sera to *S. typhimurium* lysogenized with phage P22 and its IgM and IgG fractions were adsorbed with heated cells of a nonlysogenic strain of *S. typhimurium* and tested for slide agglutination against heated cells of lysogenic and nonlysogenic strains. The factor O-1 was demonstrable both in whole serum and in the IgG fraction but not definitively in the IgM fraction (Table 1). The results suggest that IgG molecules have definite activity for antigen 1; they differentiate similar determinant groups with different linkages in the adsorption reaction.

**DISCUSSION**

Our comparative studies on the antibody response to enterobacterial O antigens confirm that a characteristic response to these antigens is early production of IgM antibody that consistently predominates until a later period when the antibody titer begins to decline (7, 15, 18, 23). These results suggest that some components of the antigen strongly elicit IgM antibody or interfere with subsequent synthesis of IgG antibody. Thus, the proposed inhibition of synthesis of IgM antibody by IgG protein (4) is relatively ineffective in immunization with *Salmonella* O antigen. It has been shown previously that a significant amount of early IgG antibody in response to *C. albicans* was found in addition to early IgM antibody, and the ratio of IgG to IgM antibody for *C. albicans* was remarkably higher than that for *Shigella flexneri* in the hyperimmune serum under the same immunizing procedure (6, 7). This has been true for the antibody responses to *Staphylococcus aureus* (Fukazawa, unpublished data) and to streptococcal group-specific bacterial vaccines (13). It is possible, therefore, that in addition to other factors affecting the antibody production.
response during immunization, such as time, dosage, route, genetic constitution of the animal (3), and serological method for antibody detection (8, 16), the immunochemical constituents in the antigen complex also play an important role in the antibody response. Since the mode of the antibody response is a complex phenomenon, analyses of the influence of the nature of the antigen on antibody induction may serve to assist in the resolution of the mechanism of the antibody response.

The complete somatic antigen extracted with cold trichloroacetic acid is a complex macromolecule composed of polysaccharide, lipid, and protein. The LPS fraction extracted with phenol-water consists of a polysaccharide component,
firmly bound lipid A, and a small amount of peptide (11). These two antigens were compared in their antibody-inducing capacity with respect to the types of antibodies produced. The data show that the antibody responses to these immunogens differed qualitatively as well as quantitatively. The LPSP elicited high levels of agglutinins associated with a considerable amount of IgG antibody, whereas the LPS induced only a low level of agglutinins which were exclusively IgM antibody. These results suggest that the protein component in the antigen complex might play an important role as a carrier for antibody induction, especially for IgG antibody after initial IgM induction. It has been reported that purified protein antigens usually stimulate a synthesis of IgG antibody after the early IgM has decreased (1, 2, 9). These findings support our suggestion on the role of carrier protein in antibody response. The higher IgG antibody response to LPSP antigen in the later period than that to somatic antigen at this time might be due to an effect of a higher dose of LPSP.

There is evidence that gram-positive microbial antigens elicit consistently more IgG antibody than do gram-negative bacteria when determined by agglutination (6, 13). As a result of various studies (14, 25), it is apparent that the cell walls of gram-positive and gram-negative bacteria differ considerably in their chemical compositions. The cell walls of gram-negative bacteria are more complex; they contain a higher percentage of lipid than do gram-positive bacteria. The immunological role of lipid in the antibody response is still unknown. Our results show that IgG antibody was produced at later periods of immunization only when a large amount of antigen was given. Therefore, a change of antibodies from IgM to IgG classes requires the necessary antigenic stimuli in addition to time. Since IgG antibody was not induced in response to LPS antigen in spite of strong stimulation, the role of the lipid moiety as a carrier, if any, is not to induce IgG antibody. LPS relatively free of protein can be prepared by a procedure of extraction with aqueous ether (17). These preparations of LPS have been shown to be highly antigenic. It has been further shown that LPS extracted with aqueous ether is distinct from the phenol-water product in that the former contains a smaller quantity of firmly bound lipid (11). It is possible, therefore, that the proportions of protein to lipid in the complex macromolecules of the LPSP and LPS preparations may explain the differences in immunogenicity. It is further suggested that the protein moiety in the complex is an active carrier in facilitating induction of both early IgM and late IgG antibodies. Lipid in the antigen complex may compete with or inhibit this action of the carrier protein, thereby inhibiting induction of IgG antibody in the primary stage of the antibody forming process.

As a comparison of the specificity between IgM and IgG antibodies, adsorption experiments were performed on each antibody fraction to detect antibody to O antigen 1, which was produced by lysogenizing Salmonella typhimurium with P22 phage (27). The results suggest that the IgG fraction contains antibody activity for antigen 1, whereas IgM antibody molecules have little or no activity for antigen 1; possibly they may be removed by adsorption by similar determinants because of cross reactivity.

LITERATURE CITED


