haptoglobin frequencies among Caucasian populations in various parts of the world. In general, the 1-1 type frequency has been in the 10-18% range. In the present study the control group 1-1 frequency was 12% while that of the patients with rheumatoid spondylitis was increased to 22%. There is roughly a 10-20% probability that this is a chance correlation and therefore this difference cannot be called significant. Almost the same figures pertain to the comparison of the patients with rheumatoid arthritis and rheumatoid spondylitis; that is, the latter have an increased frequency of 1-1 phenotype, decreased 2-1 and increased 2-2 phenotype. The p value here is roughly at the 10% level.

Studies of haptoglobins in disease have generally been concerned with states of red blood cell destruction during which the haptoglobin level is known to be diminished(8) or studies of chronic inflammatory disorders in which increases in amount of circulating haptoglobin have been documented(9,11). There are few investigations other than that on rheumatoid arthritis which have attempted to relate haptoglobin phenotypes to specific diseases. Studies of diabetic patients in Canada(12) and of hemophiliac patients in Scotland(13) have not demonstrated correlation with haptoglobin phenotypes. In the present study, the borderline significance found suggests that further evaluation of large populations could ultimately demonstrate significant correlations. It is also likely that haptoglobin subtyping utilizing the method of cleaving the polypeptide chain of the native haptoglobin would be of interest (5).

Summary. It was found that in 45 patients with rheumatoid spondylitis, 22% had a 1-1 haptoglobin type, 47% a 2-1 and 31% a 2-2 phenotype. These results were compared with published phenotypes of a normal population and a population with peripheral rheumatoid arthritis. Differences of borderline significance were observed.

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Received September 3, 1963. P.S.E.B.M., 1963, v114.

Differences in Antibody Response of Rabbit to Enterobacterial Antigen After Intravenous and Subcutaneous Injection with Adjuvant.* (28775)

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Recently, Kunin *et al*(1) described an antigen common to many species of enteric bacteria. It was shown that antisera against *E. coli* 014 and a few other serogroups cause agglutination of red blood cells modified with this common antigen (CA) from *Escherichia, Aerobacter (Klebsiella), Salmonella, Shigella,* and *Proteus.* CA has several unusual fea-

* Study supported by research grant from Nat. Inst. of Allergy and Infectious Dis., U.S.P.H.S.

tures. CA antibodies in *E. coli* 014 antiserum do not precipitate CA antigen nor produce bacterial agglutination, nor is this antigen-antibody system operative in latex agglutination(1,2). Further, antisera obtained by intravenous immunization of rabbits with numerous enteric bacteria possessing CA do not contain CA antibodies in high titer. In contrast, as shown in the present investigation, such strains readily engender CA antibodies after injection with Freund's adjuvant into the footpad. The results of the antibody response to feeding of these microorganisms are also presented.

Materials and methods. Smooth strains of *E. coli* 014, 0111, 026, *S. typhimurium,* and *S. sonnei* were grown on brain veal agar in Kolle flasks for 18 hours at 37°C. The growth was suspended in 25 ml of phosphate buffer (pH 7.3; Difco). These suspensions of living bacteria were used for feeding purposes within one hour of harvest. Others were heated for one hour at 100°C in a waterbath. Supernates were obtained after centrifugation at 23,500 *g* for 20 minutes, and were kept frozen until used. The sediment of bacterial cells was resuspended to the original volume in phosphate buffer.

Albino rabbits weighing between 2 and 3 kg were used for immunization with heated bacterial suspension, supernate, or resuspended bacterial cells. Antigen was injected intravenously or into the footpad. For the latter procedure, 0.2 ml of either antigen or an equal mixture of antigen and Freund's adjuvant (Difco) was injected into each of the footpads. The amounts of antigen and schedules are given below. For control purposes, Freund's adjuvant alone was administered.

For feeding of living bacteria the following procedure was employed. Water was withheld for 18 hours prior to feeding; the animals were offered a mixture of 10 ml of the viable suspension and 30 ml of tap water. Only after this material was ingested was additional water given. The suspension was fed daily for 10 days. The animals were housed in isolation rooms. Rabbits fed the identical strain were kept in individual cages in a single room. All cages and feeding utensils were autoclaved prior to use.

Blood specimens were obtained from all animals prior to, during, and after immunization. The serum specimens were kept frozen until used.

Titration of O antibodies was carried out by means of the hemagglutination test previously described(2,3). Briefly, serum in serial 2-fold dilutions (0.2 ml) was mixed with rabbit red blood cells (0.2 ml of 2.5% suspension) that had been modified with supernate (1:10) of heated bacterial suspension. The mixtures were incubated in a waterbath at 37°C for 30 min, and the resulting hemagglutination was read grossly after centrifugation at 1300 *g* for 2 min. The O specificity of the antibodies was ascertained by the fact that hemagglutination was not inhibited by supernates from heterologous enteric bacteria containing CA antigen (see below).

CA antibodies were titrated by means of the hemagglutination inhibition test as follows. To serum in 2-fold serial dilutions (0.2 ml) equal volumes of CA from bacteria other than *E. coli* 014 or of buffer for control purposes were added. The mixtures were incubated for 30 min at 37°C. Rabbit erythrocytes (0.2 ml) that had been modified with supernate (1:10) of a heated suspension of *E. coli* 014 were then added. The mixtures were incubated for 30 minutes at 37°C, and the resulting hemagglutination was read as described above. The elimination of hemagglutination or reduction in hemagglutinin titer by heterologous antigen is indicative of the presence of CA antibodies. In control experiments it was shown that the amount of CA used as inhibitor reduces the titer of the corresponding antibody in *E. coli* 014 antiserum by more than 95%.

Results. In the first series of experiments the antibody response to *E. coli* 0111 antigen injected by different routes and with or without complete Freund's adjuvant was studied. Groups of 4 to 6 rabbits each were immunized as follows: (a) supernate from heated suspension (dilution 1:2, 2 ml) was given intravenously at 3 to 4 day intervals for a total of 3 injections; (b) mixture of

undiluted supernate and complete Freund's adjuvant (total of 0.8 ml) into the footpads at weekly intervals for a total of 4 injections; (c) undiluted supernate in adjuvant as above into the footpads once; (d) mixture of bacterial suspension and adjuvant into the footpads at weekly intervals for a total of 4 injections; (e) undiluted supernate (total of 0.8 ml) into the footpads at weekly intervals for a total of 4 injections. The results of the titration of antibodies against CA and O antigen prior to, during, and after immunization are plotted in Fig. 1. The mean titers of sera from all animals of each group are presented.

Fig. 1 shows that either a single injection or repeated injections of *E. coli* 0111 antigen and Freund's adjuvant resulted in formation of antibodies in high titer against both O antigen and CA. Administration of antigen into the footpad without Freund's adjuvant was less effective. In contrast, intravenous injection resulted only in a minimal and temporary increase in CA antibody titers, although the O antibody response was of the same order of magnitude as that effected by administration of antigen with adjuvant. Control experiments revealed that neither complete adjuvant alone nor *S. aureus* antigen with adjuvant engendered CA antibodies.

Additional experiments were carried out with CA from other enterobacteriaceae, and similar results were obtained. For example, intravenous immunization of rabbits with *S. typhimurium* supernate failed to induce the formation of antibodies against CA; the titers remaining unaltered during the 8 week period at a level of 1:20. In contrast, titers of CA antibodies ranging from 1:640 to 1:5120 were obtained after immunization via the footpad with the identical antigen and complete adjuvant. Other enteric bacteria studied with similar results were *E. coli* 026 and *S. flexneri* type 3.

In another series of experiments complete Freund's adjuvant (total 0.8 ml) was injected into the footpads of rabbits and *S. typhimurium* supernate (2 ml of a 1:2 dilution) was administered intravenously 1 and 4 days later. During the ensuing weeks the titer of antibodies against CA did not change

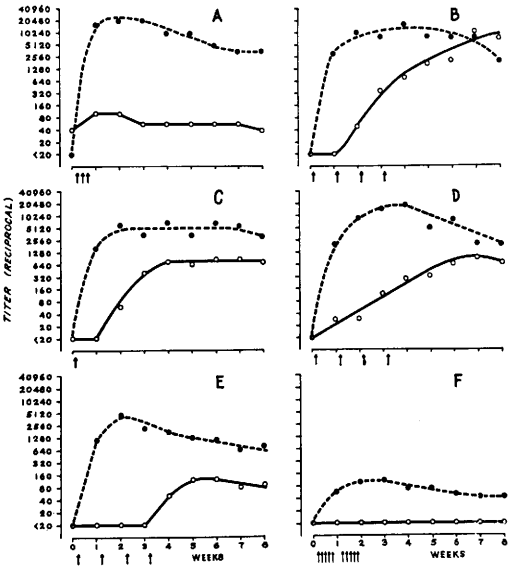

FIG. 1. Titers of CA and O antibodies in groups of rabbits after injection (A to E) or feeding (F) of *E. coli* 0111 antigen. CA antibodies, solid line; O antibodies, broken line; ↑, antigen administration; A, supernate given intravenously (3 injections); B, supernate and complete Freund's adjuvant injected into footpads (4 injections); C, supernate and adjuvant injected into footpads (1 injection); D, suspension and adjuvant injected into footpad (4 injections); E, supernate injected into footpad (4 injections); F, feeding (10 doses).

in any of the animals (mean titer 1:30), but mean O antibody titers increased from 1:40 to 1:15,000. These results suggest that after intravenous administration enough CA is not deposited at the site of the adjuvant.

To determine whether the mycobacterial component of complete adjuvant is essential for enhancement of antibody response to CA, the following experiment was carried out. Rabbits were given 4 weekly injections of 0.2 ml of mixtures of *E. coli* 0111 supernate and incomplete Freund's adjuvant into each of the footpads. The antibody titers increased 4-fold one week and 32- to 64-fold 3 weeks after immunization. When comparison is made with the antibody response following injection of antigen alone into the footpad (Fig. 1E) it is evident that the administration of antigen and incomplete adjuvant resulted in an earlier and a better antibody response. Similar results were obtained with *S. typhimurium* antigen.

Finally, the antibody response after feed-

ing of enteric bacteria was studied. Groups of rabbits were fed viable cells of either *E. coli* 014, *E. coli* 0111, or *S. sonnei*. Antibody determinations on serial blood specimens revealed that feeding of *E. coli* 014 engendered CA antibodies in relatively low titers. It is of interest that, for unexplained reasons, there was no increase in the titer of homologous O antibodies. In contrast, feeding of *S. sonnei* or *E. coli* 0111 suspension failed to engender CA antibodies, whereas O antibody titers increased more than 4- to 8-fold (Fig. 1F). These results, then, indicate that orally administered *E. coli* 014, in contrast to certain other enterobacteriaceae also containing CA, elicits the formation of CA antibodies in the rabbit.

Discussion. The present investigation has revealed that antibodies against a common antigen (CA) of various enteric bacteria, such as *E. coli* 0111 and 026, *S. typhimurium, S. sonnei,* and *S. flexneri,* are produced after injection into the footpad, with or without Freund's adjuvant, but not, or only to a minimal extent, after intravenous administration of bacterial suspension or its supernate. In contrast, O antibodies are readily produced by both methods. The poor CA antibody response after intravenous injection of antigen, observed in the present study, is in accord with the fact that numerous diagnostic enterobacterial antisera thus produced do not contain CA antibodies in high titer. Both incomplete and complete Freund's adjuvant enhance antibody formation. The mode of action of Freund's adjuvant, although explored for many years, has not been fully elucidated. Delay in antigen absorption and attraction of antibody forming cells appear to be important. When complete Freund's adjuvant is used, hypersensitivity also may play a role, for delayed hypersensitivity reaction enhances the antibody response to an unrelated antigen(4). The mycobacterial component contributes to experimental production of certain auto-immune diseases(5). Freund's adjuvant, of course, is not the sole material that enhances antibody formation. Recently, Adler and Fishman(6) reported that, for unexplained reasons, diffusion chambers increase antibody production, even when the saline filled chamber is inserted after injection of antigen. The poor CA antibody response seen after intravenous administration of enterobacterial antigens from bacteria other than *E. coli* 014 raises the question as to the distribution of the antigen or its fragments in various tissues and cells in relation to the route of injection. *In vivo* alteration of antigen, too, has to be considered, for Cohn (7) has shown that *E. coli* after residence within granulocytes is less antigenic than after residence in macrophages.

It remains unexplained why CA in *E. Coli* 014 and a few other serogroups is antigenic upon intravenous administration and the seemingly identical antigen in other enteric bacteria is not. Kunin recently succeeded in isolating CA(8), and further progress on the nature of CA in different microorganisms can be expected.

Information on the biologic significance of CA and its antibody is incomplete, but it was shown that this antibody is present in human serum and in gamma globulin preparations from various countries(9). It was also observed that an increase in CA antibody titers occurred during convalescence of children with enterobacterial enteritis(9). In view of these findings it appears justified to explore further the conditions under which these antibodies are formed in man, and to determine what effect, if any, these antibodies have on the course of infection due to bacteria containing this unusual antigen.

Summary. The antibody response of the rabbit to administration of various enteric bacteria containing both O antigen and an antigen (CA) common to numerous enteric bacilli was studied with the following results: (1) Administration into the footpad of antigen from *E. coli* 0111 and 026, *S. typhimurium,* or *S. flexneri,* together with Freund's adjuvant, resulted in formation of both CA and O antibodies in high titer. Even a single injection of antigen and complete adjuvant proved to be effective. Complete adjuvant was more effective than incomplete adjuvant. (2) Antigen (supernate) alone injected into the footpad also engendered CA antibodies, although the titers were considerably lower than those obtained after administration with

adjuvant. (3) Intravenous injection of these antigens did not result in production of CA antibodies in high titer, but O antibody titers were similar to those achieved with adjuvant. (4) Intravenous administration of *S. typhimurium* antigen into animals injected previously with Freund's adjuvant into the footpad, failed to elicit CA antibody production. (5) Feeding of viable *E. coli* 014 led to a moderate CA antibody response. In contrast, feeding of *S. sonnei* or *E. coli* 0111 engendered O antibodies but not CA antibodies.

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Received September 3, 1963. P.S.E.B.M., 1963, v114.

Erythrocyte-Modifying Capacity and Serological Reactivity of Cell Components of Human Mycoplasma (PPLO) Strains. (28776)

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Quantitation of antibody to mycoplasma organisms and determination of serological interrelationships among PPLO (pleuropneumonia-like organisms) have usually been determined with complement-fixation(1-4), agglutination(5-7), or, more recently, with indirect fluorescent antibody(8-10), latex-fixation(11), or gel diffusion(12) procedures. Direct and indirect hemagglutination and hemagglutination-inhibition tests have only been found useful in detecting antigen and estimating antibody levels against pathogenic avian(13,14), bovine(15), and porcine(16) PPLO.

The absence of a rigid cell wall containing polysaccharide or mucopolysaccharide in this group of organisms(17,18) has made the recovery of substances capable of sensitizing erythrocytes more difficult. Most investigations on isolation of serologically active fractions from mycoplasma have been carried out with the agent of contagious bovine pleuropneumonia, *Mycoplasma mycoides* (19-23).

Human strains of mycoplasma have been subjected to fractionation procedures in an effort to identify cell components participating in serological reactions and to determine whether these fractions would sensitize erythrocytes sufficiently to detect PPLO antibody in an indirect hemagglutination procedure.

Materials and methods. PPLO. The following human types and designated strains were used: *M. hominis,* type 1 (PG-25), obtained from J. S. Bailey, George Washington Univ.; *M. hominis,* type 2 (Campo), *M. fermentans,* type 3 (PG-18), *M. salivarium,* type 4 (PG-20), and *M. pneumoniae* (Eaton-FH strain) obtained from Dr. R. M. Chanock, NIAID, NIH; *M. hominis,* type 2 (07) and a saprophytic strain, *M. laidlawii* obtained from Dr. M. F. Barile, Division of Biologic Standards, NIH.

Cultivation. Ten liters of liquid media consisting of 9 parts of Difco PPLO broth, 1 part of 25% yeast extract, and 1% Difco PPLO serum fraction were used for all organisms except the PG-25 and Eaton PPLO. For these 2 strains, 10 liters of media containing 7 parts of broth, 1 part of 25% yeast extract, and 2 parts uninactivated horse