libitum-fed controls whether provided with chow or the high carbohydrate diet. These "restricted" animals had slightly lower proportion of carcass fat and higher proportion of water and ash. 3. Adult male rats when provided with the high carbohydrate diet for only 2 hours daily, ate less than *ad libitum*-fed controls and failed to gain weight.

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A Humoral Antibody Associated with Endotoxin Resistance.* (29196)

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The endotoxins of the gram-negative bacteria are highly toxic, but considerable resistance or tolerance to their pyrogenic and other effects may be induced easily by a series of injections of endotoxic preparations in experimental animals(1) or by infections in man(2). In many of their physiological effects, the endotoxins derived from different bacterial species are quite similar. Animals injected with a particular endotoxin become resistant generally not only to the homologous endotoxin but to serologically unrelated endotoxins derived from other bacterial species. Moreover, the resistance cannot be correlated with specific antibody titer(3) and may be induced in agammaglobulinemic subjects(4). Thus, a classical antibody or antitoxin response has not been invoked to explain endotoxin resistance. The mechanism responsible for endotoxin resistance has been considered generally to be an accelerated nonspecific phagocytosis of endotoxin by the reticuloendothelial system (RES). This concept was based primarily upon the reversal of endotoxin resistance by blockade and other studies associating endotoxin resistance and the functional activity of the RES.

Recent evidence has implicated also a humoral factor. Freedman reported that significant endotoxin resistance could be achieved by passive transfer of serum although he did not invoke a specific immunological mechanism to explain his results(5). Greisman, Carozza and Hills showed further that plasma from resistant animals conferred significant resistance in recipient subjects prepared by RES blockade and postulated that an opsonin may be involved in endotoxin resistance(6). Lastly, Watson and Kim(7) have demonstrated a notable degree of specificity in endotoxin resistance, in contrast to earlier work which indicated a lack of serological specificity. By means of extensive pyrogenic cross resistance tests they obtained results compatible with the presence of non-reciprocal cross-reactive antigens in different endotoxins. These findings and other ancillary evidence led these investigators to propose that endotoxin resistance was related to a classic immune response.

The present study was undertaken to detect the postulated antibody involved in endotoxin resistance by a classical serological

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procedure. Rabbits were injected with bacterial cells of one species designed to induce endotoxin resistance and then attempts were made to detect elevated levels of antibody against endotoxins derived from serologically unrelated species. The results indicated that such increased antibody levels may be detected by complement-fixation. Their rapid decline with time and swift elevation by a booster injection coincided with changes that might be expected in endotoxin resistance.

Materials and methods. Immunization of rabbits to induce endotoxin resistance. Young adult rabbits were injected intravenously with heat killed and formalin (0.5%) preserved saline suspensions containing 10^9 cells per ml. The injections were given on 8 successive days with gradually increasing doses from 0.2 ml of the suspension on the first day to 0.5 ml on the eighth. A booster dose consisted of one subcutaneous injection of 0.5 ml. The organisms used as a source of cells included *Salmonella typhosa* strain 0901, *Salmonella virginia* and *Escherichia coli* of serotype 086 obtained from the Walter Reed Army Institute of Research collection, *E. coli* strains of serotype 055 and 0111 from the Division of Laboratories, Minnesota Dept. of Health, *E. coli* serotype 08 from the Microbiology Dept., Merck Sharp and Dohme Research Laboratories, and *Chromobacterium violaceum* 9694 of the National Collection of Type Cultures, London. When purified lipopolysaccharides (product of Difco) were used rather than whole cells, rabbits were injected intravenously on 6 successive days with increasing amounts ranging from 5 μg in the first injection to 20 μg in the sixth.

Serological procedures. Complement-fixation(8), bactericidal(9), and agglutination (10) tests were performed by standard procedures. The lipopolysaccharide endotoxin preparations used as antigens in the complement-fixation test were Boivin preparations obtained from Difco; 200 μg of the lipopolysaccharide material was used since that amount was lacking in significant anticomplementary activity and found to give optimal reactivity. Epidemic typhus antigen, lot 732 B, was obtained from Dr. David B. Lackman, Rocky Mountain Laboratory, Hamilton, Mont. Endotoxin preparations by the Westphal procedure and obtained from Difco were not as reactive generally as Boivin preparations and were not used usually except for the endotoxins derived from *E. coli* 08 and *Ch. violaceum* prepared by Dr. O. Westphal and obtained from Dr. Dennis Watson.

Serological test results. The results obtained with the sera of serial bleedings from one rabbit after completion of a series of injections for 8 consecutive days with saline suspended cells of *S. virginia* and with sera of subsequent bleedings obtained after a booster dose administered 65 days after completion of the initial injections are given in Table I. Comparable results were obtained with sera of another rabbit subjected to the same dosage. The results indicated a 4-fold or greater rise in titer against the 7 endotoxin preparations tested and a 16-fold rise against the epidemic typhus antigen with the serum collected 4 days after the injection and significant but smaller rises against the *E. coli* 014 and *S. typhosa* 0901 endotoxins on the first day after completion of the injection series. The booster injection elicited no detectable agglutinin response against the homologous organism but easily detectable rises in levels of complement-fixing antibody against the 7 endotoxin preparations tested.

Although the 2 rabbits injected with *S. virginia* cells responded with at least a 4-fold rise in complement fixation titer against *S. typhosa* 0901 endotoxin, the bactericidal antibody titer of their sera against *S. typhosa* 0901 did not rise above pre-immunization levels. That this result was not a reflection of the greater sensitivity of the complement-fixation procedure for detection of antibody against the O specific polysaccharide of the organism involved was shown simply by comparative tests with an antiserum against *S. typhosa* 0901. This serum gave an approximate titer of 10,000 in the bactericidal reaction against the homologous organism, an agglutination titer of 1280, and a complement fixation titer with the Boivin endotoxin of *S. typhosa* 0901 of less than 300.

Variable responses were obtained in rabbits injected with cells of species other than

TABLE I. Agglutinin Response to the Homologous Organism and Complement-Fixing Antibody Response to Heterologous Endotoxins and Epidemic Typhus with Sera of a Rabbit Injected with Cells of *S. virginia*.

Sera	Bleeding dates	Agg. titer vs *S. virginia*	C.F. titers with endotoxins derived from: *E. coli* 014	*S. typhosa* 0901	*S. typhimurium*	*E. coli* 0111:B4	*E. coli* 055	*S. abortus equi*	*Sh. flexneri*	*Epid. typhus*
Preimmuniz	11/22/62	<20	<3	<3	—	<3	<3	<3	<3	12
Postimmuniz										
1st day post	12/11/62	1280	6	6	—	—	—	—	—	—
4th " "	12/14/62	640	12	12	12	12 or >	24	12	12	192
18th " "	12/28/62	320	3	6	—	—	—	—	—	—
55th " "	2/ 5/63	80	<3	<3	—	—	—	—	—	—
Booster	2/15/63	<20	<3	<3	<3	<3	<3	<3	<3	—
1st day post booster	2/16/63	<20	<3	<3	<3	—	—	—	—	—
4th " "	2/19/63	<20	3	12	12	6	24	6	6	—

— = not done.

S. virginia. Rabbits injected with *E. coli* 0111 gave a marked rise in complement-fixing antibody titer against the homologous endotoxin, but only a slight rise (greater than 2-fold) against both *E. coli* 055 endotoxin and epidemic typhus antigen. Sera from animals injected with *E. coli* 014 showed 4-fold rises or greater when tested against endotoxins derived from *E. coli* serotypes 055 and 0111, *S. typhosa* 0901, *Salmonella enteriditis, Salmonella typhimurium* (both Boivin and Westphal preparations). Rabbits injected with cells of *E. coli* 08 demonstrated a marked rise in complement-fixation titer (5-fold or greater) against *Ch. violaceum;* reciprocally, rabbits injected with cells of *Ch. violaceum* responded similarly against the endotoxin preparation derived from *E. coli* 08. Sera of both rabbits obtained after the injection series, compared to preimmunization control specimens, showed evidence of slightly increased reactivity (2-fold or less) against Boivin preparations derived from *S. typhosa* 0901, *S. typhimurium, S. enteriditis,* and *E. coli* 055. On the other hand, the serum of a rabbit subjected to a series of injections with *E. coli* 086, compared to a preimmunization control specimen, did not show increased complement-fixing activity with endotoxins derived from 8 different species. Whether this lack of reactivity is peculiar to *E. coli* 086 or to a particular rabbit was not ascertained.

A rabbit subjected to a series of injections with *Sh. flexneri* endotoxin gave a marked rise in titer when tested against the homologous endotoxin, from less than 3 to 192 or greater. A substantial rise in titer from less than 3 to 24 was noted in tests against *E. coli* 0111 endotoxin, but only very moderate rises, from less than 3 to 3, against other heterologous endotoxins derived from *S. typhosa* 0901, *S. typhimurium, Salmonella abortus equi,* and *E. coli* 055.

In general, therefore, significant rises in complement-fixing activity were noted against heterologous endotoxins as a result of injection procedures with killed cells or endotoxins that are capable of inducing endotoxin resistance.

Absorption studies. Reactive serum such as the 12/14/62 specimen of Table I was absorbed with heat killed and washed cells of *S. virginia.* Tested against *S. typhosa* 0901 endotoxin, there was no loss of activity as a result of the absorption, but neither did a portion of the serum sample absorbed similarly with *S. typhosa* 0901 show any loss of activity against *S. typhosa* 0901 or serologically related *S. typhimurium* endotoxin. Human group O red cells coated with heated (100°C for 1 hr) *S. typhosa* 0901 endotoxin was ineffective also in reducing the serum titer of this serum against *S. typhosa* 0901 endotoxin.

Discussion. As a result of a series of injections with heat-killed cells of one gram-negative bacterium or of purified endotoxin, significantly enhanced complement-fixing activity was observed against many heterologous, apparently serologically unrelated endotoxins. Although the rabbits injected were not tested for pyrogenic resistance, the injections undoubtedly induced considerable resistance to the pyrogenic and other effects of endotoxin. Similar injections by other investigators have accomplished this result(5). The enhanced complement-fixing activity was noted after completion of the injections and 4 days thereafter (Table I). It disappeared by the 65th day and was recalled easily by a single injection. This time sequence in the appearance and disappearance of complement-fixing activity against endotoxins derived from many different bacterial species may be correlated, therefore, with established facts related to endotoxin resistance. These include its apparent non-specificity, rapid development, short duration, and recall by a single injection(7). Finally, the enhanced complement-fixation activity against *Rickettsia prowazeki* (epidemic typhus) antigen was of particular interest since experimental animals may be protected against the toxic effects of heat-killed suspensions of *R. prowazeki* by repeated injections not only of these organisms, but also with gram-negative bacteria such as *S. virginia*(11).

The question may be raised naturally whether complement-fixing antibodies are directly responsible for endotoxin resistance. Both Greisman *et al*(6) and Watson and Kim(7) have postulated that specific humoral endotoxin inhibitors, behaving as opsonins, may be involved in the generalized RES stimulation that accompanies pyrogenic tolerance. Whether or not complement-fixing activity against endotoxins may be equated with opsonic activity remains to be determined, but it is associated certainly with the postulated opsonins. Moreover, its lack of identity with classical "O" antibody and with specific bactericidal antibody suggests a meaningful association.

The mechanism leading to elevated levels of complement-fixing antibody against a host of heterologous endotoxin preparations as a result of injection with a single endotoxin is not known. Either a common antigenic determinant in different endotoxins or a non-specific stimulation of antibody producing cells by endotoxin with predetermined specificity, in accord with the concept of clonal selection(12), represent obvious possibilities. The two mechanisms are not mutually exclusive and both may be operative. The ineffectiveness of whole cells in removing complement-fixing antibody by absorption nullified attempts to determine the serological specificity of the complement-fixation antibody and thereby determine if a common antigen were involved in eliciting complement-fixation antibody against a wide variety of organisms. Whatever its nature, the antigen involved in these reactions is a very inefficient absorbent.

A common antigen in the *Enterobactericeae* has been described recently by Kunin who has demonstrated by hemagglutination that antibodies against this antigen may be elicited particularly by *E. coli* 014(13). It was also reported that *E. coli* 014 rabbit antiserum failed to function bactericidally against heterologous strains(14), and in this respect the failure of *S. virginia* antiserum to kill heterologous *S. typhosa* in this study was similar. On the other hand, the *E. coli* 014 antiserum did not demonstrate unusual reactivity in the complement-fixation reaction. Further studies will be required, therefore, to explain the relationship of the antigen described by

Kunin and the complement-fixation results obtained in our study.

These complement-fixation studies showed evidence of reciprocal cross-reactivity between *Ch. violaceum* and *E. coli* 08. On the other hand, pyrogenic cross-tolerance tests indicated that animals tolerant to *Ch. violaceum* endotoxin were equally tolerant to *E. coli* 08 endotoxin, but that *E. coli* 08 endotoxin tolerant animals showed little tolerance to *Ch. violaceum* endotoxin(7). This lack of association between pyrogenic tolerance and complement-fixation antibody might have resulted from the fact that in our studies animals were injected with cells of the whole organism rather than with purified lipopolysaccharides; the former may have a broader specificity than its purified lipopolysaccharide.

Further studies are needed to determine the significance of the association between complement fixing antibody and endotoxin resistance. In accord with the original observations of Morgan(15), it is generally agreed that antibodies against the O specific polysaccharides of the *Enterobacteriaceae* are not involved in endotoxin resistance. The complement-fixation antibodies observed in this study are similarly unrelated to the O specific antibodies. Specific agglutinin titers against the homologous organism were not associated with the complement-fixation activity observed against heterologous endotoxins (Table I). Moreover O specific antibodies are easily absorbed from sera and they possess marked bactericidal activity(16). These attributes were not observed with the complement-fixing antibodies directed against purified lipopolysaccharides. Growing experimental evidence suggests that endotoxin resistant animals possess a dual mechanism that includes both the RES and a humoral factor which might prepare the endotoxins for their phagocytosis and loss of toxicity in resistant animals(6,7). Such a humoral factor might possess complement-fixing activity particularly in view of the enhancement of phagocytosis by complement(17).

Summary. Rabbits, subjected to a series of injections with cells or purified lipopolysaccharide of one organism designed to render them endotoxin resistant, produced elevated levels of complement-fixation antibodies against otherwise serologically unrelated endotoxins. The apparent non-specificity of the response, its temporal appearance, and recall suggested the possibility that these antibodies may be related to a humoral factor involved in endotoxin tolerance.

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