Effects of Immunization with Ethanol-Soluble Enterobacterial Common Antigen on *in Vivo* Bacterial Clearance and Hematogenous Pyelonephritis[1] (36998)

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Representatives of all genera within the Family Enterobacteriaceae share a common antigen (CA) which was described by Kunin, Beard and Halmagyi in 1962 (1) and which could be demonstrated by the indirect hemagglutination test. It is difficult to correlate these initial findings with some of the work which followed, for in their attempts to purify and characterize chemically this CA, the final product obtained failed to modify erythrocytes for hemagglutination. Subsequent work by Whang and Neter (2) showed that antigenic material in the heat-killed supernatant material derived from various enteric bacteria could be attached to erythrocytes which thereby became agglutinable in the presence of homologous as well as heterologous anti-CA sera. Furthermore, hemolysis could be demonstrated in the presence of complement, but neither bacterial agglutination nor precipitation could be shown in the presence of antibodies versus CA. Additional studies showed that the O antigen interfered with the antigenicity of CA and this led to ethanol fractionation to separate O from CA, which renders CA antigenic (3).

Studies on the biological significance of this complex antigen–antibody system via phagocytic experimentation revealed that CA antibodies opsonize enteric bacteria and also modified latex particles coated with this antigen (4, 5), suggesting that antibodies against CA might play a role in protection. Gorzynski, Ambrus and Neter (6) showed that passive immunization resulted in slight transient protection of mice against experimental *Salmonella* infection. Domingue *et al.* (7) reported that immunization with the heat-killed supernatant CA followed by ethanol-soluble CA protected rabbits against experimental hematogenous and retrograde pyelonephritis. Although specificity of protective activity was shown in the pyelonephritic experiments, the vaccine (heat-killed supernatant) contained endotoxin. Because of the nonspecific protective action of endotoxin, it was therefore deemed necessary to evaluate the role of immunization with the ethanol-soluble fraction CA only, and unrelated to priming with the supernatant material derived from boiling bacterial cells for 1 hr. We have here studied the effects of immunization with ethanol-soluble CA on *in vivo* bacterial clearance and on the development of experimental hematogenous pyelonephritis.

Materials and Methods. Common antigen preparation. As in previously described procedures (3), *Salmonella typhimurium* was grown on brain veal agar (Difco) in Kolle flasks for 18 hr at 37°. The growth was suspended in hemagglutination buffer (Difco) and boiled for 1 hr. Following centrifugation at 23,500*g* for 20 min, the supernatant fraction (HKS) was exposed to ethyl alcohol (final concn, of 85%) for 18 hr. The mixture was centrifuged; the supernatant fraction (containing CA) was air-dried and reconstituted in distilled water to one-fifth the original volume. The ethanol-insoluble material (O antigen) was washed three times in 85% ethanol, air-dried, and reconstituted to its original volume.

Serum titration: Indirect hemagglutination (IHA). Serum was obtained from all rabbits before immunization, within 1 wk prior to bacterial challenge, and at sacrifice. Hemag-

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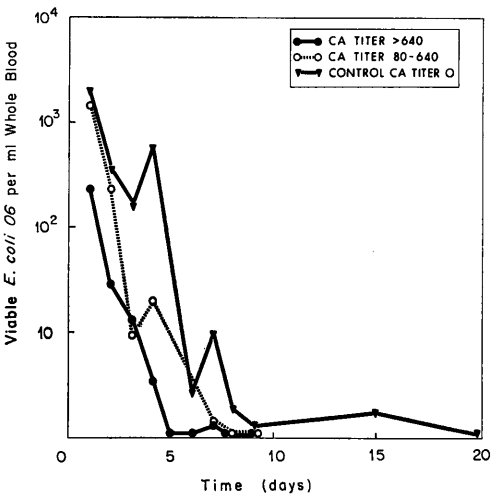

FIG. 1. Clearance of *E. coli* 06 from blood in immune versus nonimmune animals.

glutinin titers were obtained for CA (utilizing HKS of *Escherichia coli* 04), O antigen of *E. coli* 06 in all animals, and for *S. typhimurium* 0 in immunized animals prior to bacterial challenge. Sheep erythrocytes were exposed to the test antigen for 30 min at 37°. Following three washings in hemagglutination buffer, 0.2 ml of 2.5% suspension of sensitized erythrocytes was added to 0.2 ml of serial dilutions of the test serum. After incubation for 30 min at 35°, the test mixtures were centrifuged at 2000 rpm for 2 min and hemagglutination was read as the sediments were shaken. In addition, many of the titers were determined by the microhemagglutination method after it was established that comparable titers were obtainable. The highest serum dilution producing visible hemagglutination was taken as the IHA titer.

Immunization. Rabbits (adult New Zealand whites) of either sex, weighing 4–7 lb, were actively immunized by intravenous injection on alternate days of 1 ml of 5 × concentrated CA derived from *S. typhimurium*. Titers were checked at various intervals during and at 1 wk after the last immunizing dose, and animals were boosted if IHA titers had not reached 1:80.

Hematogenous challenge. Rabbits were fed 2.5% oxamide mixed in their diets (8) for 12 days. During the feeding of oxamide, microscopic intraductal calcifications were found. When the administration was stopped, calcifications were soon absent. Pyelonephritis thus initiated, however, continued. On Day 10 of feeding, the animals were injected intravenously with 1.7 to 5 × 10^8 washed viable organisms of *E. coli* 06 strain obtained from a patient with acute pyelonephritis.

Postchallenge treatment. Blood cultures were obtained by daily cardiac puncture following hematogenous challenge. Various dilutions of heparinized whole blood were added to trypticase soy agar and incubated for 48 hr at 35° to determine the viable count per milliliter.

Following hematogenous challenge, animals were sacrificed at 2 and 4 days, and 1, 2, 3 and 4 wk.

Urine was removed by needle puncture of

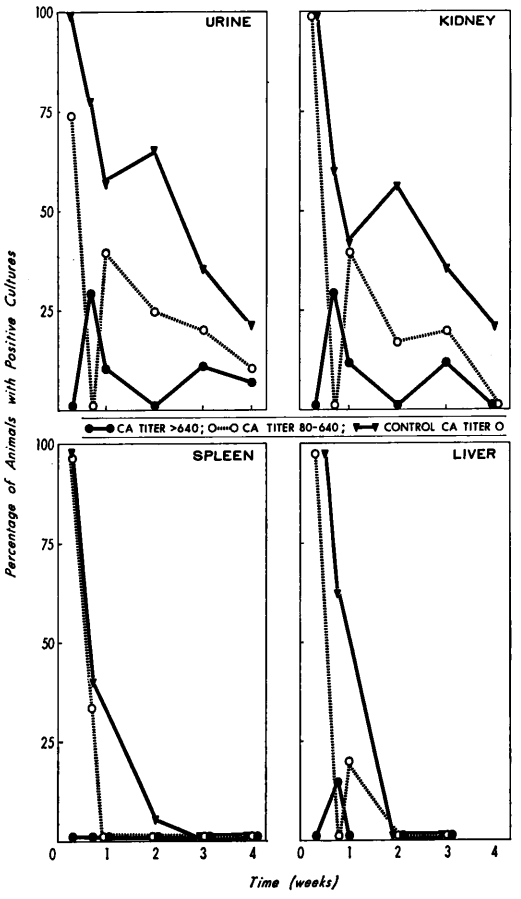

FIG. 2. Percentage of animals with positive culturral results in immune versus nonimmune animals.

the bladder for quantitative cultures. Blood was removed by needle puncture of the inferior vena cava and cultured as described above. Kidneys, spleen and liver were removed aseptically.

The individual tissues were weighed and examined for gross pathology. A section of both kidneys was cut for histopathology and a weighed portion of all tissues was then ground by hand with sterile normal saline, using a glass tissue homogenizer. Pour plates were made of the suspension, utilizing trypticase soy agar incubated at 35° for 24–48 hr; the viable count was expressed per gram of tissue. Representative bacterial colonies were picked and biochemical patterns as well as slide agglutination tests with homologous antiserum were used as criteria to insure identity of the organism with the original challenge strain. Thin sections of both kidneys of each rabbit were stained with hematoxylin–eosin and examined for evidence of renal pathology, without knowledge by the pathologist as to identity of specimens.

Results. Qualitative blood culture results indicated that the percentage of positive blood cultures in immunized and control animals were similar to each other until Day 8, at which time all animals with high CA titers $>$ 1:640 yielded negative cultures, all animals with lower titers becoming negative at 9 days; control animals did not completely clear their blood stream until 21 days. None of the immunized animals had positive blood cultures 9 days postchallenge, whereas 10% of the controls remained positive until Day 21 postchallenge. When quantitative blood culture results are considered (Fig. 1) rapid clearance becomes quite obvious in immunized animals (on a direct titer-related basis).

Spleen culture results are summarized in Figs. 2 and 3. Although the percentage of positive cultures was essentially identical for control and immunized animals during Days 2 and 4 (Fig. 2), all spleen cultures from the immunized ones were negative at 1 wk, whereas a small proportion of control animals remained positive at 2 wk. It was of interest that all high-titer immunized animals had negative spleen cultures at each sacrifice interval. Quantitative results in Fig. 3 further substantiated the rapidity of clearance in the immunized animals: those with low titers

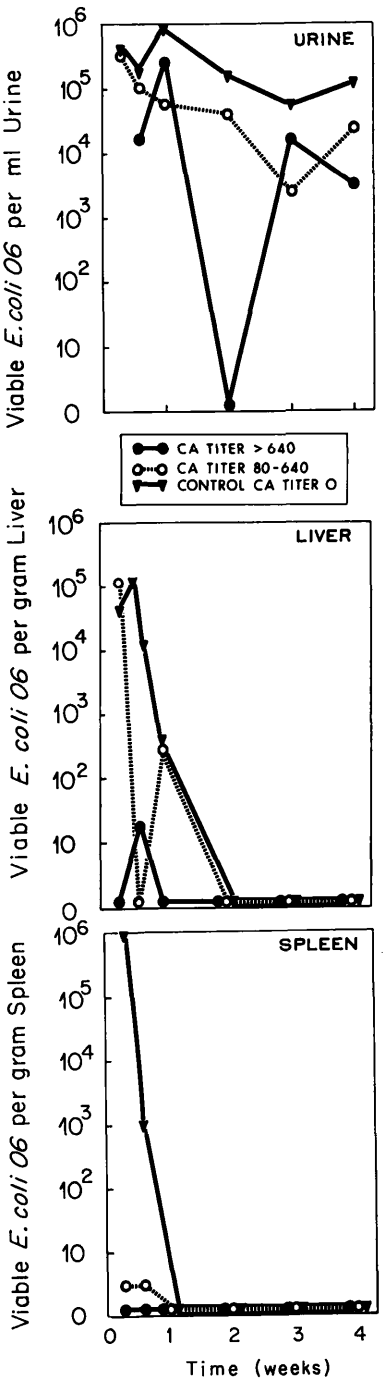

FIG. 3. Quantitative cultural results in immune versus nonimmune animals.

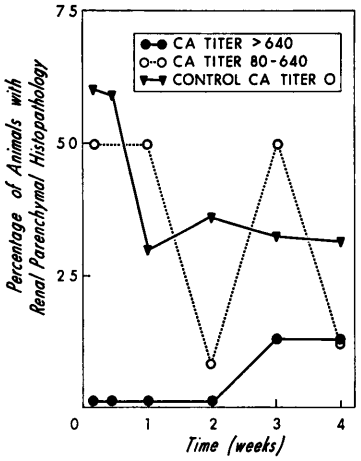

FIG. 4. Renal parenchymal histopathology in immune versus nonimmune animals.

which were positive at 2 and 4 days had counts in the range of 0–10 organisms/g, whereas control cultures were in the range of 2.0×10^4 to 2.2×10^6 organisms/g at 2 days, and 10^2 to 1.3×10^4 at 4 days; three control cultures were negative at this time interval.

Qualitative liver culture results are shown in Fig. 3. Again, a more rapid clearance of challenge organisms was demonstrated in immunized animals on a direct titer-related basis, with the quantitative results presented in Fig. 4 further substantiating these findings. It should be noted that the apparent discrepancy in low-titer animals at 1 wk was due to one animal with a positive culture; four were negative at this interval.

Urine culture results (Figs. 2 and 3) showed that, although the trend of clearance remained the same as for the blood, spleen and liver, a significant proportion of immunized animals had bacteriuria at all intervals. Even at 4 wk postchallenge, 8% high-titer, 11% low-titer immunized animals, and 25% control animals yielded positive urine cultures.

Figure 2 also shows qualitative kidney culture results with the same trend of clearance as noted in other tissues, except that complete clearance is not present in immunized animals until after 4 wk; 25% of controls were positive at that time. Clearance appeared to be directly related to CA antibody titer. In general, when the quantitative renal data were analyzed, 46% of the control animals versus 9% (high titer) and 36% (low titer) immunized animals showed positive findings of challenge organisms in renal tissue over the 4 wk period. The number of animals with $> 10^3$ organisms/g of kidney tissue was also greatest in control animals (30% versus 9% high-titer and 20% low-titer immunized animals).

Based on the histopathology, the kidneys were placed into three general categories: (a) acute pyelonephritis; (b) histologically not remarkable; (c) abnormal, but with nonspecific alterations. Frequently, morphologic changes were limited to only one of a pair of kidneys. With bilateral involvement, often there was a difference in degree of change observed.

Acute pyelonephritis was characterized by extensive though patchy destruction of renal parenchyma by an acute inflammatory process, noted particularly in the cortex, but present also in pelvic and calyceal areas. Collecting tubules in the medulla frequently contained neutrophils.

Another group of kidneys examined revealed no histologic abnormalities.

In the third group of kidneys, evidence of pyelonephritis was equivocal, yet the organs were not histologically normal. In most of this group, chronic pyelitis was present; it ranged in severity from only a sprinkling of lymphocytes and plasma cells to severe chron-

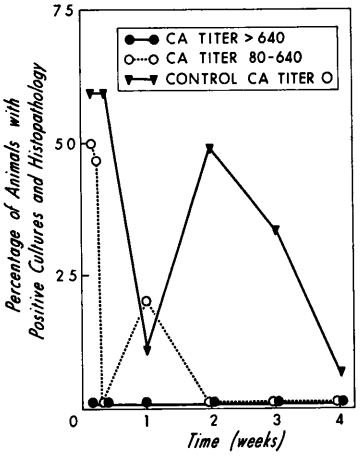

FIG. 5. Comparison of positive renal cultures with parenchymal histopathology in immune versus nonimmune animals.

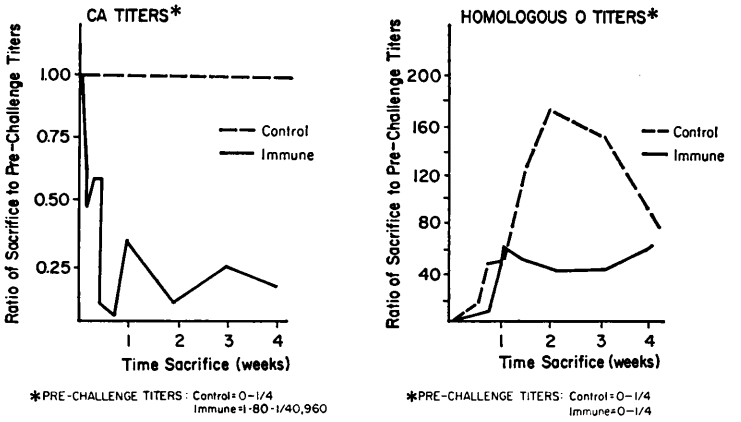

FIG. 6. Antibody titers in immune and control animals.

ic inflammation. A frequent observation was focal accumulation of lymphocytes and plasma cells in the interstitial areas of the cortex. This was interpreted as an abnormal, though nonspecific change. Occasional kidneys contained focal scar formation, characterized by parenchymal collapse, fibroblastic proliferation and chronic inflammatory infiltrates. Focal tubular calcifications and crystalline deposits were each frequently noted in this group. It must be emphasized that the changes noted in this third group of kidneys are not diagnostic of pyelonephritis, but can occur in other pathologic states. The presence, however, is abnormal and indicates that pathologic processes have occurred, especially since kidneys from animals fed oxamide but receiving no challenge bacteria or immunization had no evidence of histopathology.

In the analysis of histopathologic data, if animals with only evidence of parenchymal pathology (excluding pyelitis) are considered (Fig. 4), there was essentially no difference in control and low-titer immunized animals. Animals with titers over 640 demonstrated less evidence of parenchymal disease than the other groups. The majority of control animals with pathology also had positive cultures (Fig. 5), which suggests active infection; 10% were positive even after 4 wk. Low-titer immunized animals cleared their infection rapidly and were negative by 2 wk. It is of interest that none of the high-titer immunized animals demonstrated active infection at any time interval.

The change in hemagglutination titers with time was plotted as the ratio of sacrifice/prechallenge titers (Fig. 6). Control animals demonstrated negligible titers to CA (0 to 1/4) prechallenge, and these titers did not change after hematogenous challenge with *E. coli* 06. Titers in immunized animals rapidly decreased after challenge and stabilized at approximately 20% of the prechallenge levels. It should be noted that a heterologous system (HKS of *E. coli* 04) was used in testing for CA antibodies. Neither control nor immunized animals had significant titers to O antigen of *E. coli* 06 prior to hematogenous challenge. By 1 wk, anti-O antibodies developed in both immune and controls and rapidly stabilized in the immunized group. Titers continued to rise and peaked after 2 wk in the control animals. All prechallenge immunized animals were tested for antibodies against O antigen of *S. typhimurium,* from which the CA used for immunization was obtained. The rabbits were found to have 100-fold lower O antigen titers than CA titers, suggesting only minor O antigen contamination of the CA vaccine used for immunization.

Discussion. Evidence has been obtained suggesting that immunization with CA derived from *S. typhimurium* protects rabbits against active renal parenchymal infection following hematogenous challenge with *E. coli* 06 known to contain CA.

Blood culture studies indicate a more rapid clearance of organisms in immunized animals on a direct titer-related basis. Since the anti-

body against CA is known to be opsonizing, one may speculate that increased phagocytosis is at least in part the mechanism of clearance in these animals. As would be expected, the more rapid clearance is noted in liver and spleen, major organs of the reticuloendothelial system. Although clearance is delayed in the kidneys, the trend of titer-dependent clearance is still apparent and all immunized animals are cleared by 4 wk, when 25% of controls are positive. When one combines the finding of a positive renal culture and parenchymal histopathology in which inflammatory cells are present, this clearance in immunized animals is even more dramatic. Few immunized animals had overall renal parenchymal histopathology at the time of sacrifice and only 6% (of 38 animals) of low-titer animals had positive renal cultures. These animals were sacrificed 1 wk or earlier postchallenge. All had negative cultures after 2 wk. No high-titer immunized animal (of 37 animals) had histopathology and positive renal cultures at any time interval, whereas an overall 20% of control animals (of 78 animals) fell into this category, 5% still being positive at 4 wk postchallenge.

The ethanol-soluble CA has been shown to be virtually free of endotoxin by the method of Pieroni *et al.* (9) which is based on the actinomycin D potentiation of endotoxin lethality for mice. The results of a typical experiment comparing the toxicity of the ethanol-soluble fraction containing CA to that of *E. coli* 0127:B8 lipopolysaccharide has revealed that 10 mg of ethanol-soluble fraction (nine times the usual vaccination dose) was no more toxic than 1 μg of purified lipopolysaccharide (McLaughlin and Domingue, unpublished data). Also, post immunization titers to the O antigen *(S. typhimurium)* were 100-fold less than CA titers. Therefore, the nonspecific protective role of endotoxin and anti-*S. typhimurium* O antibodies are not offered as an explanation for our findings. It should be emphasized that a totally heterologous system was employed in this study and no animal had an appreciable titer to the O antigen of the challenge organism prior to hematogenous inoculation or, in fact, after immunization. In addition, there is no known cross-reaction between *S. typhimurium* O antibodies and *E. coli* 06.

The apparent paradoxical finding of bacteriuria and negative renal cultures or absence of renal histopathology deserves mention. This finding was also noted in the study reported by Domingue *et al.* (7) and suggests that serum antibodies are responsible for protection against infection, rather than CA antibodies being secreted into the urine. This seems likely, since anti-CA globulins are of the 19S type (10), which, at the present time, have not been demonstrated in the urine. Again, as in the previous study (7), low bacterial counts were noted in the kidneys of immunized animals which had positive urine cultures, yet no renal histopathology. These results suggest contamination with tubular fluid.

In previous studies (7), vaccines used in priming injections for immunization were heat-killed supernates and contained relatively large amounts of O antigen in combination with CA and endotoxin. Other investigators demonstrated that priming with the above preparation stimulated higher titers of CA antibodies when purified CA was used for booster injections (11). Our studies have shown that, although lower titers against CA develop when partially purified vaccines are utilized for priming and booster injections, protective activity of CA antibodies is still demonstrable. The importance of this finding bears emphasis, since an ideal vaccine for human use would be devoid of endotoxin contamination and preferably contain only purified CA.

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