STUDIES ON INFECTION AND IMMUNITY IN EXPERIMENTAL TYPHOID FEVER

II. Susceptibility of Recovered Animals to Re-Exposure*

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(Received for publication, July 15, 1960)

While it is commonly believed that recovery from typhoid fever confers a fairly solid immunity, the older literature contains a number of reports of second attacks (1). Generally speaking, the second attack rate has been reported to range from 1 to 4 per cent, a figure compatible with (although by no means proving) the assumption that recovery leads to immunity. However, there are significantly higher second attack rates on record (15 per cent, (1)) high enough to raise doubts as to the assumption stated above. An outstanding recent example of such a report is that of Marmion, Naylor, and Stewart (2), who published an account of recurrences of typhoid fever in a British Royal Air Force unit in the Suez Canal Zone in 1950. Two large outbreaks, occurring in the same community within 5 months, produced 11 instances of second infections. The first outbreak was caused by phage-type J bacilli and the second by phage-type E_1 organisms, indicating that the second cases were new attacks of the disease rather than relapses of the original infection. These workers concluded that an attack of typhoid fever confers only a moderate degree of specific immunity, and explained the usual low incidence of multiple infections, at least in part, on the lack of re-exposure to typhoid bacilli.

Studies on experimental typhoid fever in progress at this institution for a number of years have confirmed and extended the findings of Grünbaum (3), and Metchnikoff and Besredka (4) that a disease can be produced in chimpanzees with clinical, laboratory, and histopathological findings closely resembling those occurring in human typhoid infections (5–7). During the course of these investigations the status of immunity in chimpanzees following recovery from typhoid fever was examined. The response of these animals to rechallenge is described in this report.

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* Presented in part before the Society of American Bacteriologists, April, 1958, at Chicago, Illinois.

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Materials and Methods

Experimental Animals.—Young chimpanzees of both sexes, weighing from 26 to 34 pounds at the time of re-infection were employed. These animals, which had recovered from typhoid infection induced 15 to 21 months earlier, were housed and maintained at Walter Reed Army Institute of Research (WRAIR) together with uninfected chimpanzees of the same age and weight range for 6 months prior to the original infection and during the period preceding the present rechallenge study.

Challenge Culture.—The stable, well known Ty2 strain of *Salmonella typhosa*, phage-type E_1 (8), was employed as the re-challenge culture. The characteristics of this strain and its infectivity for chimpanzees are described in a previous publication (7).

Challenge Procedures.—The challenge method employed was the same as that used to infect the animals initially (7). A 5 hour veal infusion agar culture of *S. typhosa* Ty2 was harvested in saline and the chimpanzees challenged orally by feeding each a banana inoculated with the desired number of organisms. The dose administered was determined turbidimetrically and by subsequent viable count. At the same time, the mouse virulence of the challenge culture was ascertained by intraperitoneal inoculation, employing saline suspensions of the organisms. LD_{50} values of approximately 3×10^6 bacilli were obtained as calculated by the method of Reed and Muench (9).

The presence of Vi and O antigens in the challenge inocula was confirmed serologically, using rabbit anti-Vi serum produced against *Paracolobactrum ballerup*, and rabbit anti-O serum produced against *S. typhosa* O-901 (7).

Clinical and Laboratory Observation of Challenged Animals.—The course of infection was followed for 28 days by daily stool and blood cultures, twice daily rectal temperatures, and close observation for changes in appetite or general condition. Blood was also drawn for sedimentation rate and for determintion of antibody, C-reactive protein, and properdin levels. Sedimentation rates, corrected for hematocrit values, were determined by the method of Wintrobe. Properdin assays were performed by the Department of Serology, WRAIR, using the technique of Pillemer *et al.* (10), while C-reactive protein levels were measured by the United States Army Medical Unit, Fort Detrick, Maryland, employing a modification of the method of Anderson and McCarty (11). Hemagglutination tests for Vi antibody were performed according to the procedure described by Landy and Lamb (12); the standard bacterial agglutination procedure was employed for the determination of O and H antibody titers (13).

Bacteriologic Procedures.—Stool specimens were cultured on duplicate SS agar plates. In addition, approximately 1 gm. of stool was inoculated into selenite-F enrichment broth, which was subcultured on SS agar after overnight incubation. Non-lactose fermenting colonies were transferred to Kligler's iron agar slants, which were examined subsequently for biochemical changes characteristic of *S. typhosa*. In those instances in which biochemical changes were atypical, verification was made by slide agglutination.

Blood was cultured by inoculating 2 ml. into 18 ml. of brain heart infusion broth, which was incubated for 7 days. As soon as bacterial growth was evident, or after 7 days of incubation in the absence of discernible growth, subcultures were made to SS agar. Colonies suspected of being *S. typhosa* were handled in the same manner as described for stool culture isolates.

RESULTS

Six previously infected and recovered chimpanzees were available for these studies. Four of these animals (2 males, 2 females) had been infected *via* the oral route with 100 billion of a phage-type T strain of *S. typhosa* by Mandel

et al. (14) at WRAIR. This culture, designated as the Illinois Carrier strain 2593, has been described previously (7). Within 5 days, these animals had shown evidence of infection as indicated by bacteriemia, development of fever, and the isolation of typhoid bacilli from their stools. Antibody responses occurred in all of the animals, with peak O titers ranging from 1:40 to 1:1280, and peak H titers varying from 1:320 to 1:5120. Two of the four chimpanzees showed a small, transient Vi antibody response with hemagglutination titers of 1:7.5 and 1:15. Within a month following challenge, all animals had recovered.

In the present study, 15 to 18 months after initial infection, these 4 recovered chimpanzees together with 2 unexposed control animals (males), were challenged orally with 100 billion *S. typhosa* Ty2. Both controls exhibited febrile response, bacteriemia, elevated sedimentation rate, C-reactive protein in the serum, lethargy, and some evidence of dehydration. On the other hand, all 4 previously infected and recovered chimpanzees were negative with respect to these laboratory and clinical observations, and therefore, were considered free of significant infection. Antibody responses which differed markedly in the two groups, are discussed in detail below.

Figs. 1 and 2 illustrate the findings on the 2 control animals which had had no previous contact with the typhoid bacillus. As shown in Fig. 1, stool cultures on control chimpanzee Mel were positive for the first 4 days post challenge, and negative thereafter. Bacteriemia appeared on the 6th post-challenge day, followed in 24 hours by fever, which rose to a peak of 103.8°F. from this animal's average pre-challenge rectal temperature of 99.9°F. Fever continued throughout the next week coincident with bacteriemia, and during this time this chimpanzee was lethargic and moderately anorectic. Temperature rapidly declined to normal levels at the time blood cultures became negative. A definite elevation in sedimentation rate was observed on the 14th and the 22nd day, dropping to the normal range by the 28th day, while marked increases in C-reactive protein were seen during the 2nd week of infection. A slight drop in properdin level was noted for the 10 day period following challenge, but this decrease was so small as to be of questionable significance. Good O and H antibody titers were present after the 1st week; Vi antibody could not be demonstrated at any time.

In the case of the other control animal (Tom), Fig. 2, stool cultures were positive during the 3 days immediately following challenge. Febrile response, which began on the 5th post-challenge day, attained a maximum of 103.2°F. as compared to this animal's average pre-challenge rectal temperature of 99.3°F., and was of shorter duration than that occurring in the case of chimpanzee Mel. One positive blood culture was obtained during the febrile period. Slight lethargy was apparent, but anorexia was not discernible. This animal, as well as the other control, showed some dehydration, as evidenced by a loose, dry skin, and moderate constipation. The sedimentation rate was elevated from the

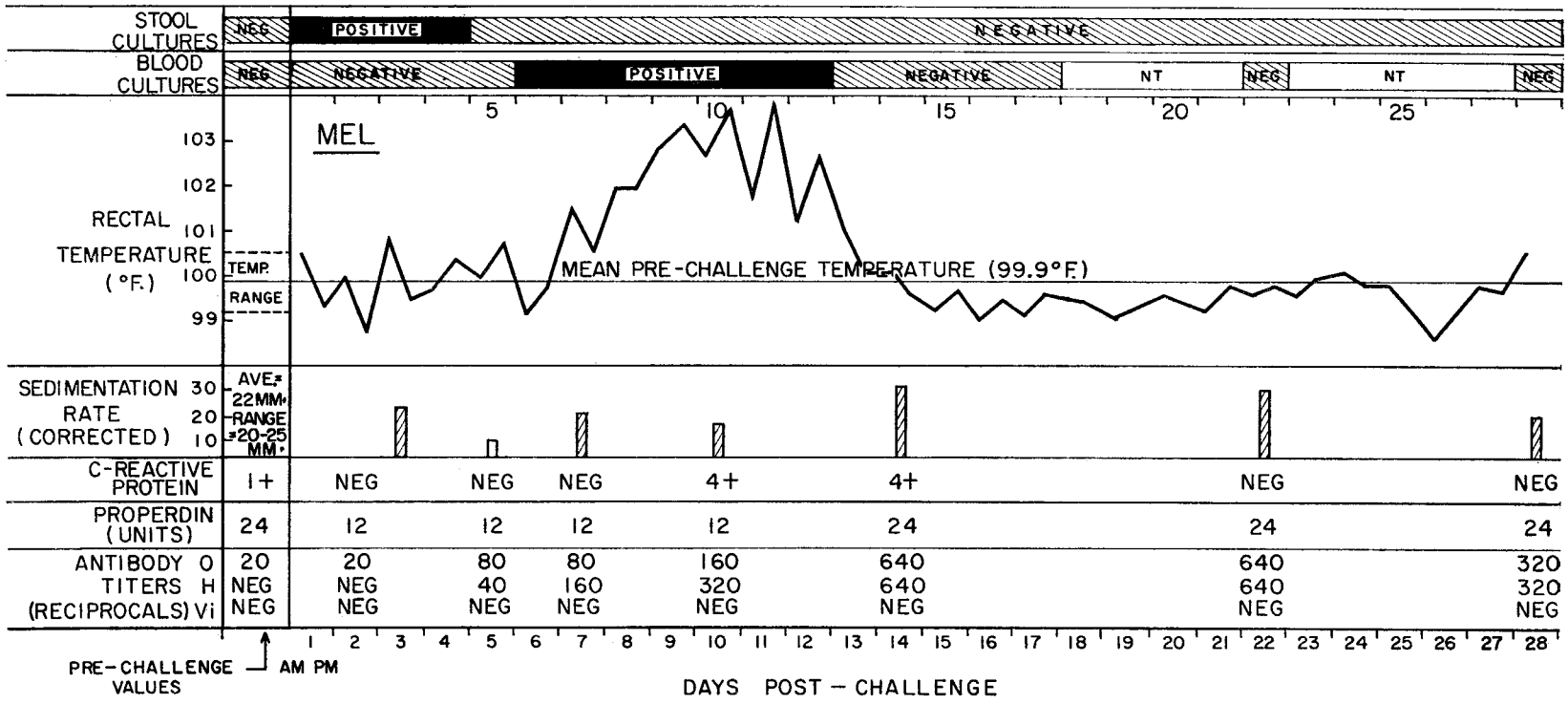

FIG. 1. Response of chimpanzee Mel (not previously infected) to oral challenge with 100×10^9 *S. typhosa* Ty2. NT, not tested.

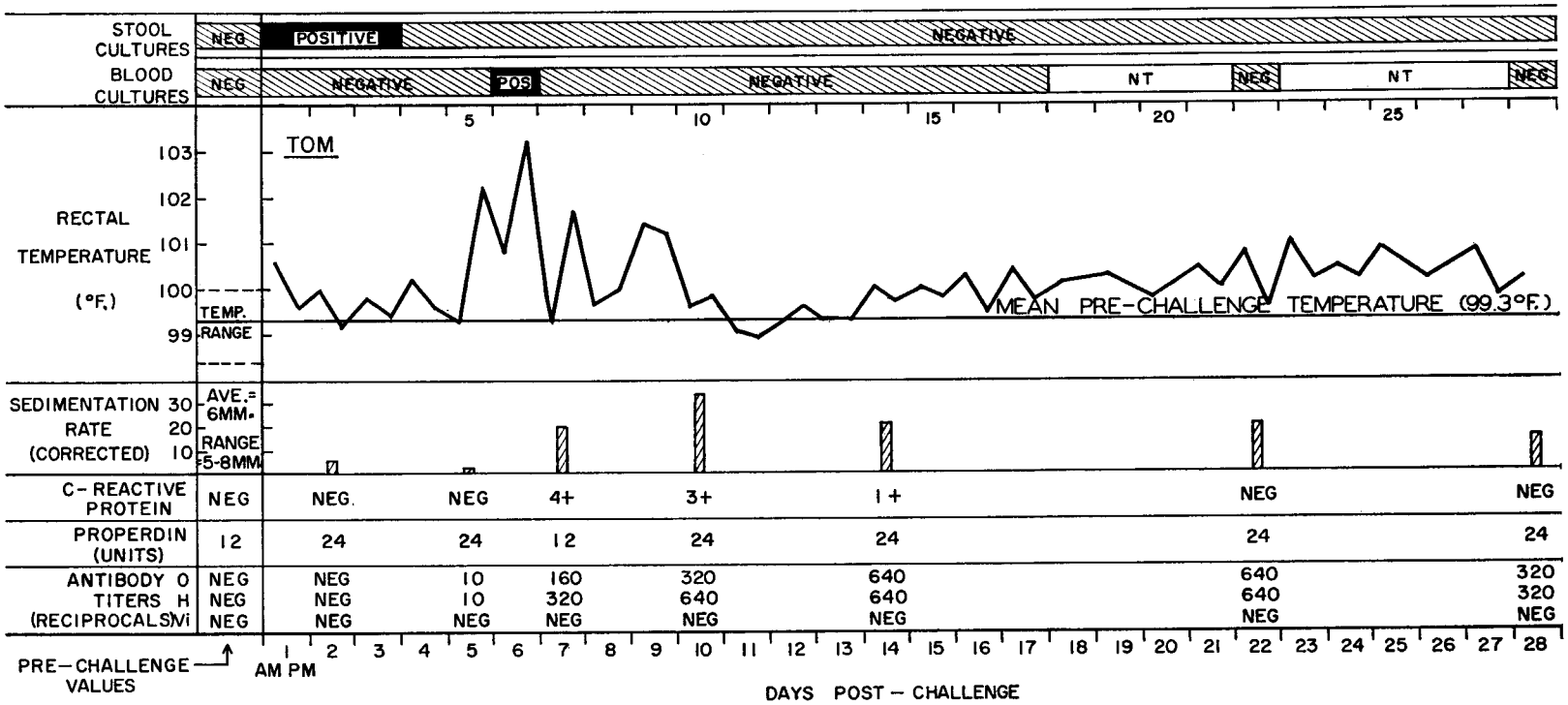

FIG. 2. Response of chimpanzee Tom (not previously infected) to oral challenge with 100×10^9 *S. typhosa* Ty2.

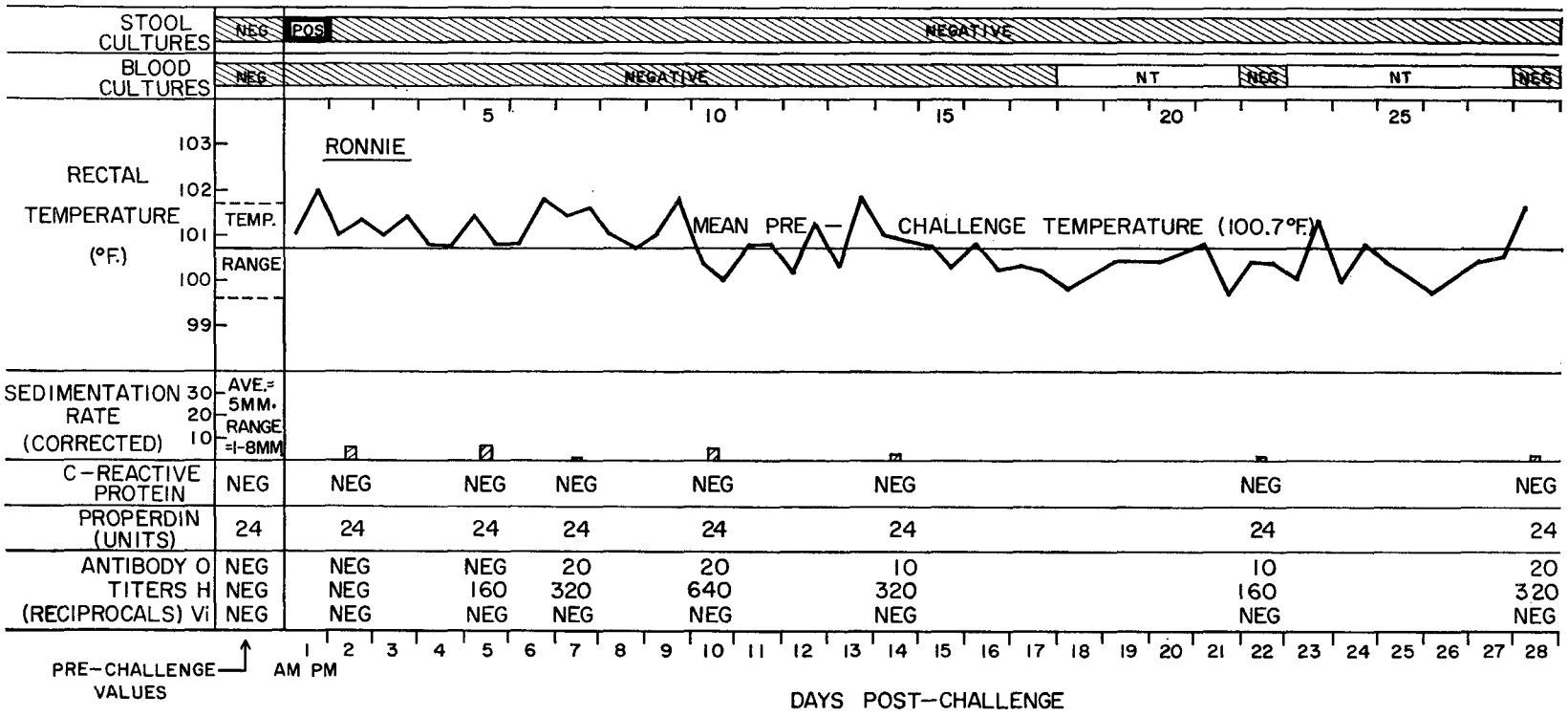

FIG. 3. Response of chimpanzee Ronnie (previously infected with 100×10^9 *S. typhosa* No. 2593) to oral challenge with 100×10^9 *S. typhosa* Ty2.

7th day onward and, like the other control animal, chimpanzee Tom showed a marked increase in C-reactive protein during the 2nd week of infection. Properdin levels, however, remained essentially unchanged. O and H antibodies were demonstrated as early as the 5th post-challenge day, and rose rapidly to peak titers (1:640) during the 2nd week. Tests for Vi antibody were consistently negative.

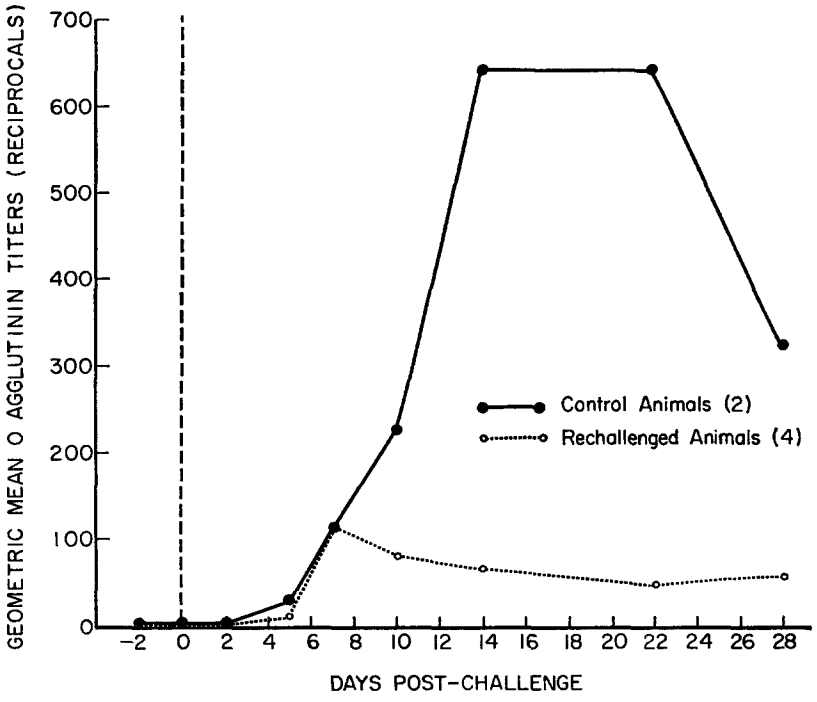

FIG. 4. O antibody titers of chimpanzees in response to oral challenge.

Fig. 3 depicts the results obtained in the case of a rechallenged chimpanzee (Ronnie). A positive stool culture was obtained only on the 1st day post-challenge and bacteriemia could not be demonstrated at any time. Febrile response was absent, the sedimentation rate was not increased, serum specimens were negative for C-reactive protein, and properdin levels were not altered. O antibody did not appear in the serum of this chimpanzee until the 7th day post-challenge and remained at a low level, in contrast to the development of a good H antibody titer as early as the 5th day. Vi titers were negative throughout. At no time did this animal show signs of lethargy, anorexia, or dehydration. The other three animals of the rechallenged group had essentially identical clinical courses. Furthermore, daily attempts to demonstrate bacteriemia were con-

sistently unsuccessful, and the other laboratory findings were similar to those obtained on chimpanzee Ronnie.

At the time of rechallenge measurable H antibody levels were seen in 3 of the 4 chimpanzees of the rechallenged group, but only one of these animals exhibited

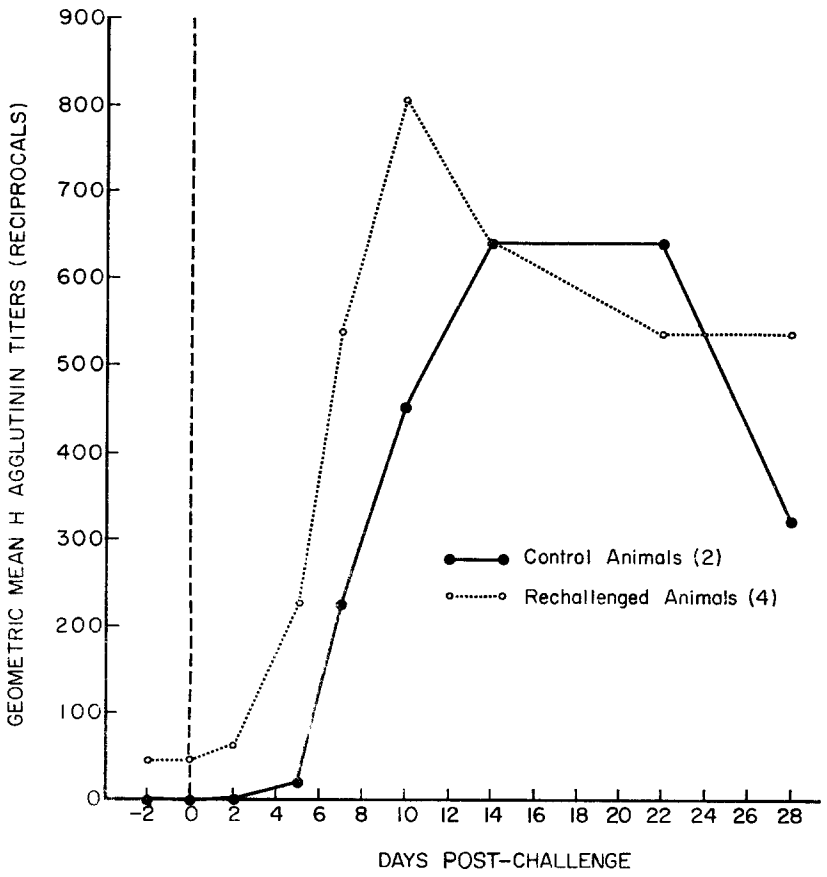

FIG. 5. H antibody titers of chimpanzees in response to oral challenge.

O antibody prior to challenge. On the other hand, except for an O antibody titer of 1:20 in one animal, pre-challenge O and H antibodies were not detected in the control group. A comparison of the O and H antibody levels of the two groups of animals prior to and following challenge is shown in Figs. 4 and 5, respectively. As seen in Fig. 4, O antibody in the control animals rose to an average peak titer of 1:640 by the 14th post-challenge day and remained at this level for 1 week before dropping. In the rechallenged chimpanzees, however, an average peak of only 1:113 was reached on the 7th post-challenge day followed by a

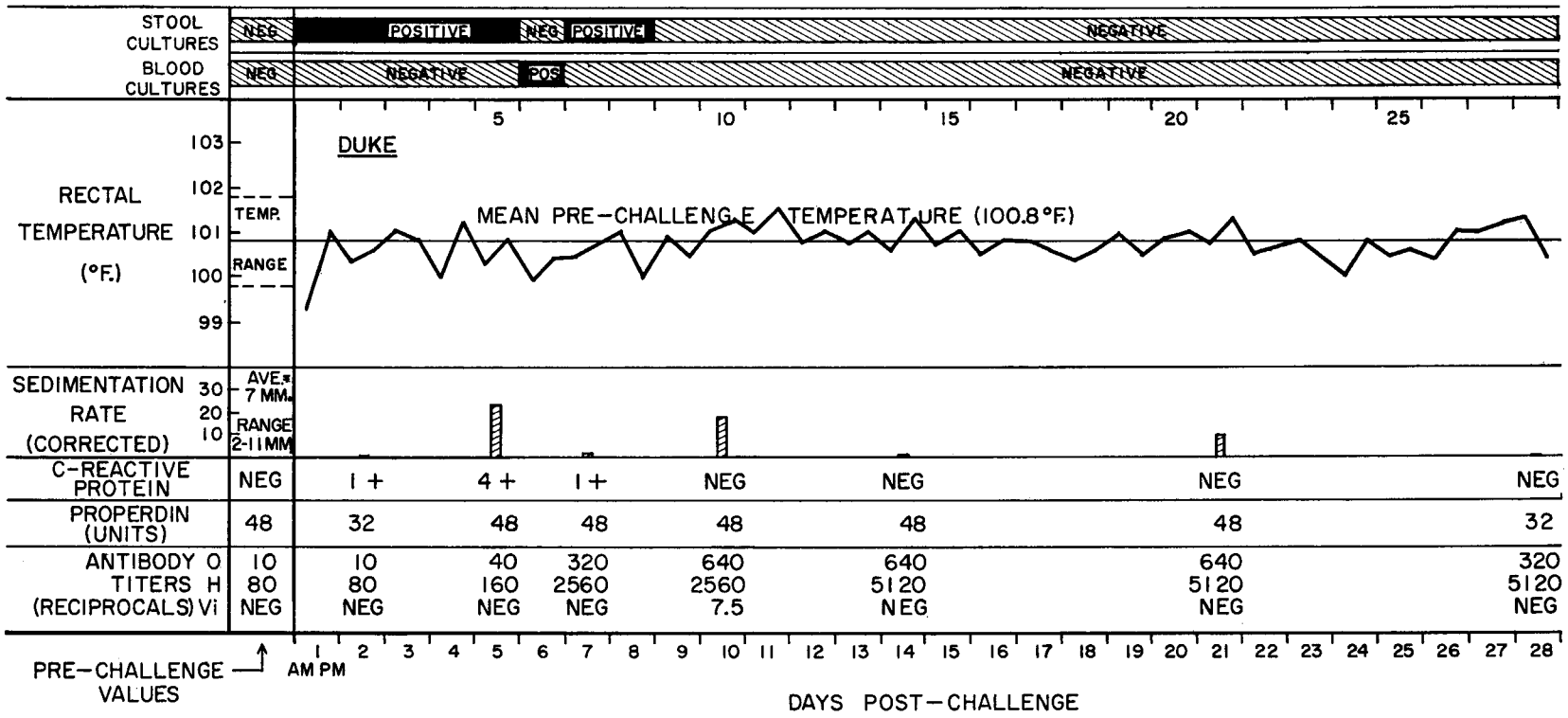

FIG. 6. Response of chimpanzee Duke (previously infected with 4×10^9 *S. typhosa* No. 2593) to oral challenge with 87×10^9 *S. typhosa* Ty2.

decline within 3 days. In contrast to these findings, Fig. 5 shows that H antibody titers for the rechallenged group attained an average maximum of 1:806 as compared to an average peak H titer of 1:640 in the control chimpanzees. It is noteworthy that peak O and H antibody titers, irrespective of their levels, were reached several days earlier in the case of the rechallenged animals.

In an attempt to determine the effect of size of the original infecting dose on subsequent rechallenge, the 2 remaining, previously infected chimpanzees (1 male and 1 female), were employed. These animals, which had recovered from initial infection produced with relatively small numbers of typhoid bacilli, were rechallenged with a large dose comparable to that employed in the experiment described above. Approximately 21 months before rechallenge, these chimpanzees had been infected orally by Mandel *et al.* (14) with 4 billion *S. typhosa* No. 2593. The administration of this inoculum resulted in bacteriemia of short duration (1 day per animal), but neither chimpanzee exhibited fever, anorexia, or lethargy. Typhoid bacilli were isolated from the stools of one of these chimpanzees during the 2nd and 3rd weeks following infection. Moderate O and H antibody responses developed in both animals and reached maxima of 1:320 during the 3rd and 4th weeks, but Vi antibody could not be detected.

Following oral rechallenge with 87 billion *S. typhosa* Ty2, a mild infection was again established in both chimpanzees. Fig. 6 illustrates the observations made on one of these two animals (Duke). Stool cultures were positive during the first 8 days following challenge, and negative thereafter. Temperatures were essentially normal, despite the presence of bacteriemia on the 6th day. An elevated sedimentation rate occurred on the 5th and 10th post-challenge day, and an increase in C-reactive protein was seen on the 5th day. The properdin level was not significantly altered. Pre-challenge O and H antibody titers of 1:10 and 1:80 respectively, rose to peaks of 1:640 for O antibody and 1:5120 for H antibody during the 2nd week. A slight transitory Vi titer (1:7.5) was seen on the 10th day. Throughout the course of the experiment, this chimpanzee was active, had a good appetite, and showed no apparent illness.

The other animal exhibited a generally similar picture. Stool cultures were positive for the first two days following challenge and bacteriemia was present on the 8th and 9th day. Alterations in sedimentation rate, properdin level, and C-reactive protein content of the serum were not significant. Pre-challenge antibody was not apparent, but antibody response to rechallenge resulted in peak titers of 1:160 for the O and 1:320 for the H during the 3rd week. Vi antibody could not be demonstrated. This chimpanzee also showed no evidence of clinical illness at any time.

DISCUSSION

The experiments described in the present investigation clearly support the concept that recovery from typhoid infection in the chimpanzee results in a sig-

nificant degree of immunity. Whether this immunity leads to complete or only partial protection of the rechallenged animal, appears to depend, at least in part, on the relative size of the successive infecting doses. Thus, when a large inoculum was used to produce infection initially, rechallenge with an equivalent number of *S. typhosa* a year and a half later was not associated with bacteriemia or clinical evidence of disease. The laboratory evidence of infection was confined primarily to antibody response. On the other hand, when a relatively small inoculum had been used to establish the infection initially, rechallenge with large numbers of typhoid bacilli approximately 18 months later resulted in readily demonstrable bacteriemia, serological response, and alterations in sedimentation rate and C-reactive protein levels. In a preliminary rechallenge study conducted previously by Gaines, Edsall, and Landy (15), typhoid fever was first established in 8 chimpanzees with inocula of 3 to 5 billion *S. typhosa* Ty2. Five to 11 months later, these animals were refractory to rechallenge with a like number of the same strain of organism. From these results, it seems reasonable to assume that re-infection may occur when the size of the rechallenge inoculum significantly exceeds the number of organisms producing the first infection. If the number of organisms involved in the original infection is small, the antigenic stimulus provided may not be enough to produce sufficient immunity to protect against subsequent exposure to large numbers of bacilli. While such immunity is not necessarily confined or related to humoral response, it has been our experience that those chimpanzees infected initially with large inocula showed a greater, more rapid antibody response than did those infected initially with smaller doses of bacilli. It is pointed out that the above assumption is based on results obtained with a small number of chimpanzees, and furthermore, that the virulence of the infecting strains, the physical state of the host, and many other factors conceivably play a role in immunity to or the occurrence of multiple attacks of this disease.

The status of immunity in man following recovery from typhoid fever, although long considered as being of a high order, has been questioned in light of the recent experiences in the British Armed Forces in the Middle East (2) and the earlier reports of second attacks (1). It is appreciated that the analogy between immunity to typhoid fever in man and in the chimpanzee is limited, but because of the similarity of the disease in these two species, it nonetheless may be instructive to compare the results of our study in chimpanzees with the findings of Marmion *et al.* in man. These investigators suggested that the occurrence of second attacks might have been due to the lack of cross-immunization between different phage types, although they stated that there was no experimental evidence to indicate such immunological specificity with respect to phage types of *S. typhosa*. Our results, however, indicate that the difference in the phage types of the two strains of typhoid bacilli was not the critical factor in the re-establishment of typhoid fever, since exposure to phage-type E_1 bacilli fol-

lowing recovery from infection with phage-type T organisms resulted in mild reinfection in one instance and resistance in another.

It is possible that the second cases reported by Marmion and his coworkers were due to a difference in the virulence of the two strains involved, although no virulence data were given. In our study, the strains of *S. typhosa* utilized for the initial and subsequent infections were of essentially the same antigenic composition and of comparable mouse virulence and, aside from phage type, differed only in the numbers of organisms employed for challenge.

Thus, it may be that, like re-infection of the chimpanzees, the second cases of typhoid fever in the British troops involved a much greater infecting dose. This is suggested by the report of the Middle East investigators that the first outbreak was milder and less extensive than the second, an observation indicating the possibility of larger infecting doses in the latter. Indeed, the extremely high incidence of second attacks (11 out of a possible 54) and the incredibly high attack rate of 34.2 per cent in the entire unit during the second outbreak, as compared to a 12.8 per cent attack rate in the first outbreak, suggest that a high proportion of the population at risk in the second outbreak was exposed to overwhelmingly large doses of typhoid bacilli. Other factors, including virulence of the strains involved and the presence of intercurrent infections or infestations may also have contributed to the occurrence of these second cases. In any event, the outbreak appears to have been unlike most other outbreaks of typhoid fever, and the more general experience of a 1 to 4 per cent second attack rate is more consistent with the assumption that in man, as apparently also in the chimpanzee, a reasonable degree of immunity follows recovery from typhoid fever.

In the present investigation, the differences in the O and H antibody curves of the control and rechallenged animals are worthy of note, since the responses observed for the latter represent booster-type reactions to antigenic stimuli, in contrast to the initial-type responses of the control group. The O antibody response of the rechallenged group was essentially the same as that seen in the controls during the 1st week following challenge. After the 7th day, however, O antibody titers of the rechallenged animals declined from the relatively low levels attained, in contrast to the sustained and striking O response in the controls. Conversely, H antibody in the rechallenged group appeared earlier, rose at a faster rate, and reached a higher level than in the control animals. These observations indicate a difference in the nature of the booster responses to O and H antigens; the development of H antibody in the rechallenged animals depicts a typical protein-type booster response, while production of O antibody in these animals represents a booster response characteristic of lipopolysaccharide antigens.

It is of interest to compare the results of this report with the findings obtained in studies on prophylactic immunization (14, 16, 17). The present work has demonstrated that under the proper circumstances, chimpanzees may be pro-

tected for at least 18 months following recovery from typhoid infection. The results of the immunization studies, to be described in detail in a subsequent publication (17), showed that chimpanzees were well protected for at least a short interval following anti-typhoid immunization. It is clear, therefore, that recovery from infection, as well as administration of vaccine, can provide demonstrable protection against subsequent challenge with viable typhoid bacilli.

SUMMARY

Chimpanzees recovered from typhoid fever induced by ingestion of large numbers of phage-type T *S. typhosa* were rechallenged approximately a year and a half later with a like number of a phage-type E_1 strain. Control animals exhibited febrile responses, bacteriemia, and other significant laboratory and clinical findings, including increases in C-reactive protein levels and sedimentation rates. All of the previously infected and recovered chimpanzees were negative with respect to the aforementioned observations, and appeared to have resisted significant re-infection. On the other hand, recovery from typhoid fever induced by smaller numbers of *S. typhosa* failed to protect completely against rechallenge with larger numbers of these organisms.

These findings indicate that chimpanzees recovered from typhoid fever may be protected against re-infection, even though the re-infecting organism is of a different phage type. Differences in the magnitude of the original and subsequent infecting inocula appear to influence the response to rechallenge.

We wish to express our appreciation to Dr. Geoffrey Edsall and to Dr. Maurice Landy for their valuable suggestions during the preparation of this manuscript.

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