

Immunological Study of Typhoid: Immunoglobulins, C₃, Antibodies, and Leukocyte Migration Inhibition in Patients with Typhoid Fever and TAB-Vaccinated Individuals

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Received for publication 22 April 1974

The development of humoral and cell-mediated immune responses to *Salmonella typhi* antigens and immunoglobulin and C₃ levels were determined in patients suffering from typhoid fever, TAB-vaccinated individuals, and appropriate controls. In 45 patients with typhoid, a significant elevation of immunoglobulin M (IgM) level was noted from the first week of illness onwards. Eighteen TAB-vaccinated persons also showed a significant elevation of IgM levels. In typhoid sera, the anti-O and anti-H antibodies were mostly 2-mercaptoethanol (2-ME) sensitive. The rise of IgM level correlated well with the 2-ME-sensitive anti-O and anti-H antibodies seen in typhoid patients. The anti-O antibodies in the TAB-vaccinated group were almost entirely 2-ME sensitive, but both 2-ME-sensitive and -resistant anti-H antibodies were detected in the TAB group. A marked increase in C₃ level was also noted in patients with typhoid. The cell-mediated immunity (CMI), as measured by leukocyte migration inhibition tests, was demonstrable in 15 of 22 patients with typhoid. On the other hand, only 8 of the 20 normal subjects, 5 of the 16 fever control cases, and 6 of the 18 TAB-vaccinated individuals gave a positive CMI. The latter three groups were comparable with each other but were significantly different from the typhoid patients. It was concluded that TAB-vaccination did not induce CMI even though it induced the development of antibodies, the latter being comparable with those of the patients with typhoid. The significance of these findings is discussed.

The nature of protective immunity in typhoid in man is not well understood. The development of the humoral immune response to O, H, and V₁ antigens of *Salmonella typhi* has been regularly demonstrated during and after typhoid fever as well as after TAB vaccination (23). A few recent studies have indicated that the antibodies may not be important in protection against this disease (16, 34). The development of cell-mediated immune response (CMI) in typhoid fever has not been studied to the best of our knowledge. The leukocyte migration inhibition (LMI) technique has been shown to correlate well with other parameters of CMI (3, 5, 11, 33). It is simple to perform and works well with particulate antigen (10). The present study describes the results of LMI in the presence of *S. typhi* antigens in patients with typhoid fever, TAB-vaccinated individuals, and appropriate controls. In addition, the study describes the immunoglobulin levels, C₃ levels, and characterization of anti-O and anti-H antibodies in typhoid patients and TAB-vaccinated individuals.

MATERIALS AND METHODS

Subjects. Forty-five fever patients with blood cultures positive for *S. typhi* were studied for immunoglobulins, C₃, and antibodies. LMI was studied in only 22 of these 45 patients. For normal base-line data on immunoglobulin and C₃ levels, 50 healthy volunteers from the staff and students of the All-India Institute of Medical Sciences, with no history of typhoid fever or TAB vaccination during at least 5 years preceding this work, were studied. The base-line data on LMI were obtained by studying the same parameters in 20 of these 50 normal subjects. The other controls for LMI included 16 patients suffering from fever due to causes other than typhoid who were admitted to the hospital and who gave no history of TAB vaccination and typhoid fever during at least 5 preceding years. The socioeconomic status of the typhoid group and the normal control group was similar, since most of the patients with typhoid were family members of the employees of the Institute. The levels of immunoglobulins, C₃, antibodies, and LMI were also studied in 18 TAB-vaccinated individuals. They had taken TAB vaccine (Central Research Institute, Kasauli) containing 10⁸ heat-killed, phenol-preserved *S. typhi* (strain TY-2) and 5 × 10⁷ each of *S. paratyphi* A and *S. paratyphi* B per ml. A 1-ml dose

was given subcutaneously followed by 0.5 ml of the same 4 weeks later and 0.5 ml every year thereafter. None of the patients gave a history of typhoid fever during the preceding 5 years.

Immunoglobulin and C₃ levels. The single radial diffusion technique of Mancini et al. (22) was used. The technique and its standardization using locally produced monospecific antisera to immunoglobulin (Ig) G, IgM, and IgA has been described (21). WHO standard no. 67/97 was used to estimate immunoglobulins. A pool of normal sera locally collected and calibrated against the standard provided by Meloy Laboratories served as standards for the estimation of C₃ levels. The results of immunoglobulin studies were expressed in WHO potency units, and the levels of C₃ were expressed in milligrams per 100 ml. As explained in an earlier communication (21), the statistical calculations were done after conversion of the values to logarithms.

O and H agglutinins. The standard Widal agglutination test was used with O and H agglutinable suspensions of *S. typhi* (Central Research Institute, Kasauli). The starting dilution of serum was 1:20, and dilutions up to 1:640 were used. The reciprocal of the last tube showing clear agglutination of the organisms was taken as the titer of the test serum. The end titer was not determined in cases where the serum gave a positive test up to a dilution of 1:640.

19 S and 7 S agglutinins. For the estimation of 19 S and 7 S, O, and H agglutinins, the 2-mercaptoethanol (2-ME) reduction technique was used. In this procedure 0.2 M 2-ME was mixed with an equal amount of test serum and incubated at 37 C for 1 h. The treated serum was again tested for O and H agglutinins by the Widal technique. The fall in titer obtained after reduction was taken as representing 19 S agglutinins, and the residual titer remaining after the reduction procedure was taken as representing 7 S agglutinins.

LMI. The method of Soborg and Bendixen (30) was followed with slight modifications. Approximately 10 ml of heparinized blood (450 IU of heparin per ml of blood) was mixed with an equal volume of 2% gelatin and allowed to sediment at 37 C for 0.5 h. The leukocyte-rich supernatant was collected and washed twice with heparinized Hanks balanced salt solution. The cells were finally suspended to a concentration of 30×10^6 to 40×10^6 per ml in Eagle minimal essential medium containing 10% fetal calf serum. Capillaries (75 mm long with an internal diameter of 1 mm) (Gelman-Hawksley, UK no. A804) were filled with the leukocyte suspension, one end was sealed with plasticine, and the capillaries were centrifuged at $250 \times g$ for 10 min to pack the cells. The capillaries were cut at the cell-fluid interface, and the cell-containing portion was deposited in locally made Mackness-type perspex chambers. The chambers were filled with minimal essential medium plus 10% fetal calf serum, with or without the antigen. In each test, four capillaries were prepared with the antigen and four were prepared without. The chambers were incubated horizontally at 37 C for 20 h. The area of migration was determined by projection and planimetry. The results were expressed as migration index, i.e., the mean of the area of migration with antigen in four

capillaries divided by the mean of the area of migration without antigen in the remaining four capillaries. A migration index of 0.8 or less was considered to be positive.

A VW strain of *S. typhi* isolated from the blood of a patient suffering from typhoid fever was used as the source of antigen in LMI. A smooth colony of the strain was subcultured over a nutrient agar slope and incubated at 37 C overnight. Growth was harvested in sterile physiological saline and washed twice with sterile saline, and the wet weight of the organisms was determined. The bacteria were then suspended in a suitable amount of sterile distilled water and ultrasonically lysed at 20 kc for 15 min. The lysed suspension was heated at 56 C for 1 h. After checking for sterility, this was used at a final concentration of 3 mg (wet weight) of organism per ml of fluid to fill the chambers.

RESULTS

There were 5 patients in the first week of the illness, 18 during the second week, 16 during the third week or later, and 5 patients during relapse. All were on specific antibiotic treatment for a varying period of time.

There was a significant increase in the levels of IgM in the patients with typhoid at all stages of illness as well as in those given TAB vaccine. There was also a statistically significant rise in the IgG level in patients after the third week or more of illness. IgA was found to be elevated in the first week, but the number of patients in the first week was so small that its significance is questionable. The level of C₃ was found to be very much elevated in typhoid patients. Persons given TAB also showed elevated C₃ levels, but not to the same extent as the typhoid patients (Fig. 1).

Anti-O and anti-H antibodies in typhoid patients were mostly sensitive to 2-ME reduction (Fig. 2). In the TAB group, anti-O antibodies were almost entirely 2-ME sensitive, whereas anti-H antibodies in almost half of the subjects showed a significant titer after 2-ME reduction (Fig. 3).

Good correlation was found between 2-ME-sensitive anti-O and anti-H antibodies and the level of IgM at weeks 1, 2, and 3 or later of illness and during relapse (Fig. 4).

In normal controls, 8 out of 20 subjects (40%) gave a positive reaction in LMI. A comparable number of individuals after TAB vaccination (33%) and in the fever control group (31%) also gave a positive response. In the typhoid fever group, 15 out of 22 subjects (68%) gave a positive LMI. The difference was statistically significant when compared with any of the three remaining groups (Fig. 5). The patients with typhoid were divided according to the time of illness at which LMI was performed (Fig. 6).

the TAB-vaccinated subjects did not differ. The patients suffering from typhoid fever and

significantly from each other with regard to O and H antibody response (Table 1).

In typhoid cases, there was a good correlation between the positive LMI and antibody response. There were a few cases in which the two responses were present independent of each other (Table 2). Most of TAB-vaccinated individuals developed the antibody response, whereas only a few exhibited LMI (Table 3).

DISCUSSION

A recent report of the World Health Organization Scientific Group has outlined lacunae in the present knowledge of the immunological aspects of typhoid fever (35). It has recommended that a detailed study of antibodies as well as the cell-mediated immune mechanisms in typhoid fever is needed to identify the most effective markers of the immune state. In the animal model of *Salmonella* infection, the role of CMI in protection is well established (19, 32). Also, some recent studies have shown that CMI (with concomitant protection) can be induced in mice only with living rather than killed vaccines (6, 9, 20), unless the killed vaccine is incorporated in Freund complete adjuvant (7, 8).

Our results show that the individuals immunized with TAB vaccine develop a consistently good humoral immune response against *S. typhi* antigens. This response was almost entirely of 2-ME-sensitive antibodies, which presumably belong to the IgM class. However, about one-half of the vaccinated group also showed a significant amount of 2-ME-resistant type of anti-H antibodies, which are presumably IgG in nature. This observation was consistent with earlier reports that killed *Salmonella* vaccines stimulate antibodies of mostly IgM type, except

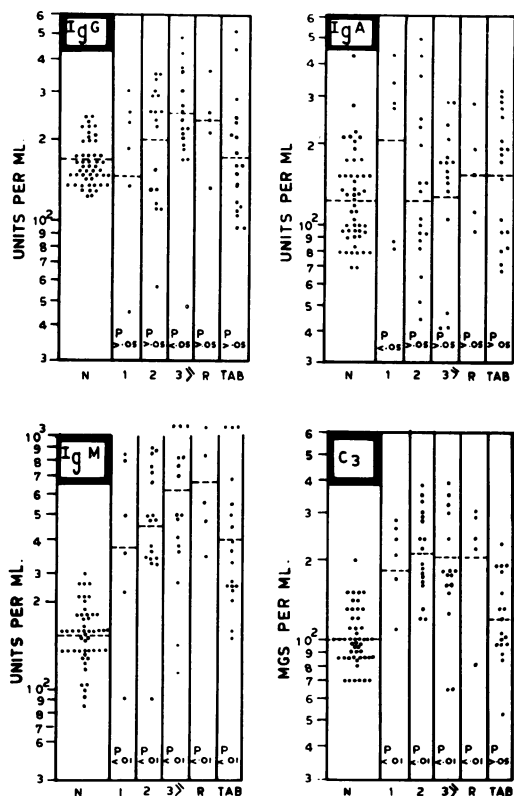


FIG. 1. Levels of IgG, IgM, IgA, and C₃ in patients with typhoid, TAB-vaccinated individuals, and controls. N, Normal controls; 1, 2, and 3, weeks of illness at which the patients were studied; R, patients studied during relapse. Immunoglobulin levels are represented in WHO potency units. P values in each column are in comparison with normal controls.

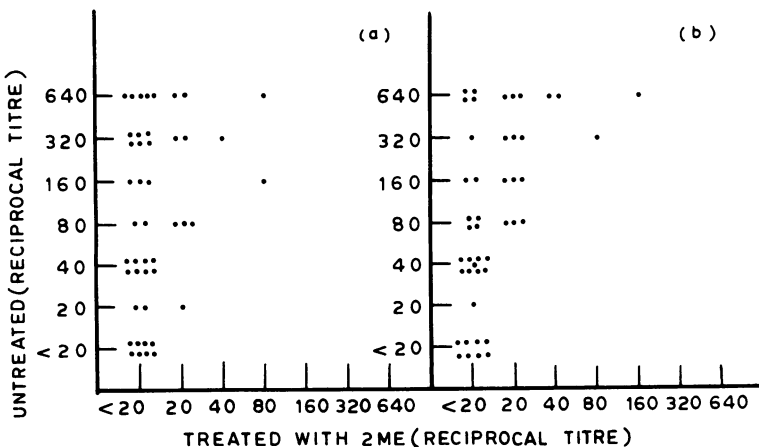


FIG. 2. Effect of treatment with 2-ME on anti-O (a) and anti-H (b) antibodies in patients with typhoid.

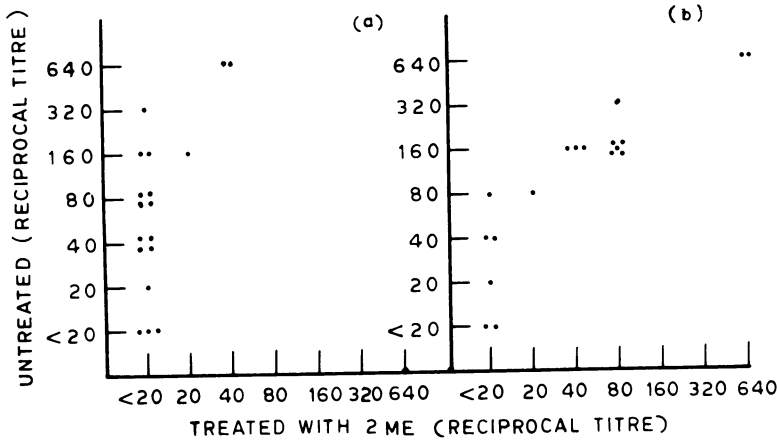


FIG. 3. Effect of treatment with 2-ME on anti-O (a) and anti-H (b) antibodies in-TAB vaccinated individuals.

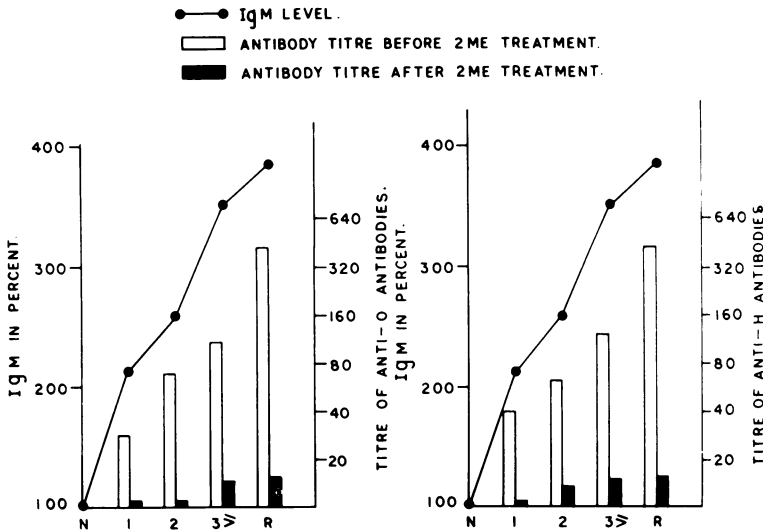


FIG. 4. Correlation of serum IgM levels and anti-O and anti-H antibodies before and after 2-ME treatment in patients of typhoid. IgM level of normals is represented as 100%. The mean IgM levels at different stages of illness are represented as percentage of normal. The symbols on the abscissa are the same as in Fig. 1.

to H antigens, against which IgG antibodies develop after intensive and prolonged immunization (1, 4, 12, 17, 31). The humoral immune response in patients with typhoid fever showed a difference from that in vaccinated normals in that during the third week or more of illness, significant levels of anti-O and anti-H antibodies of the 2-ME-resistant type develop, though the major response was still of 2-ME-sensitive antibodies. This was in contrast to the report by Pinto et al. (25) and Pinto and Dammaco (24), who failed to find any IgG anti-O antibodies in typhoid. However, the results are consistent with the report of Chernokhustova et al. (4), who found anti-O and anti-H antibodies in all the three major immu-

noglobulin classes in typhoid.

The predominantly IgM antibody response both in TAB-vaccinated individuals as well as in the typhoid group is reflected in an early and sustained rise of IgM serum levels. The rise of IgG level in the third week of illness may be a reflection of the development of IgG anti-O and anti-H antibodies that develop after sustained and prolonged antigenemia.

Immunoglobulin levels have been reported to be high in persons from developing countries (15, 18, 26, 27). The prevalence of infestations and infections in such populations has been postulated to be the cause for high immunoglobulin levels. The present finding of raised IgM levels at all the stages of typhoid fever and

elevated IgG levels during third week or later of illness gives some weight to this argument. Consistent with the fact that the complement level rises in most inflammatory conditions (2, 13), a very marked rise in C₃ serum level was noted in typhoid starting from first week of illness onwards. In a study of five cases of experimental typhoid in man, Schubert et al.

(28) also found rising whole hemolytic complement levels in all the subjects. It has been postulated that antibodies may be responsible for tissue damage in typhoid fever (14). In other conditions like systemic lupus erythematosus and glomerulonephritis, where antibodies are involved in the pathogenesis of diseases, complement levels are low (29). The finding of the

LEUKOCYTE MIGRATION INHIBITION IN TYPHOID

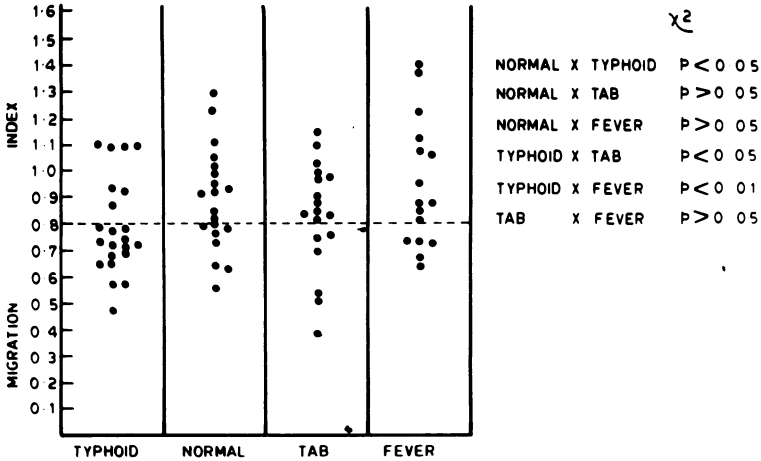


FIG. 5. Migration index, as determined by leukocyte migration inhibition test, in patients with typhoid fever and in controls.

**LEUKOCYTE MIGRATION INHIBITION TEST
IN DIFFERENT STAGES OF TYPHOID.**

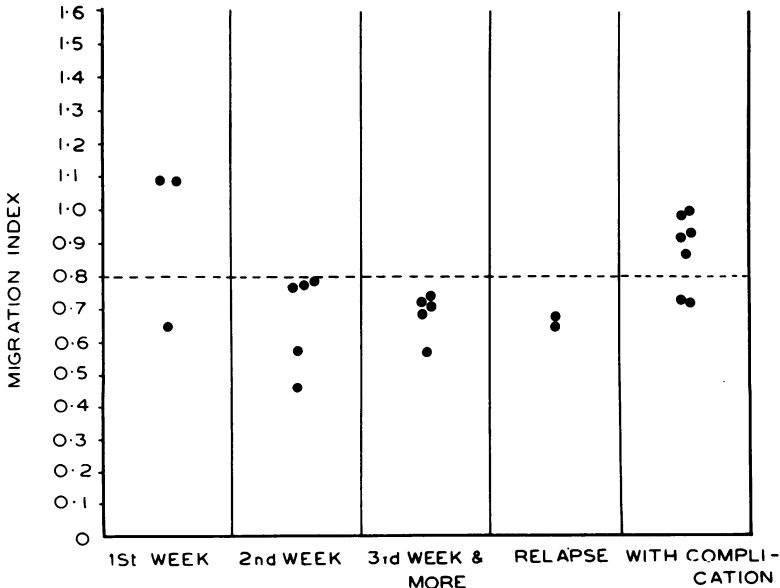


FIG. 6. Migration index at different stages of typhoid fever.

TABLE 1. *Titers of O and H antibodies in typhoid patients and in TAB-vaccinated individuals*

Titer	Typhoid ^a		TAB vaccinated	
	O	H	O	H
≥640	1	4	2	2
320	7	4	1	1
160	1	1	3	8
80	3	4	4	2
40	3	2	4	2
20	1	0	1	1
<20	6	7	3	2

^a 22 cases. Numbers indicate number of patients.

TABLE 2. *Correlation of LMI with O and H antibodies in typhoid patients*

LMI	Antibody			
	O		H	
	+	-	+	-
+	12	3	10	5
-	4	3	5	2

TABLE 3. *Correlation of LMI with O and H antibodies in TAB-vaccinated individuals*

LMI	Antibody			
	O		H	
	+	-	+	-
+	5	1	6	0
-	10	2	10	2

high levels of complement in typhoid fever may be indirect evidence against the participation of antibodies in causing tissue injury.

One of the significant findings in the present work was that a fair number of apparently healthy individuals as well as those suffering from fever due to causes other than typhoid gave a positive LMI response to *S. typhi* antigens. It has been suggested that in an endemic area, the adult population that is exposed to frequent subinfective doses of the causative organisms would show a certain degree of resistance to typhoid fever (16). The presence of positive LMI in controls may represent a degree of basal LMI in the population under study with concomitant resistance to typhoid.

It was also observed that the status of LMI in TAB-vaccinated individuals was not significantly different from those of normal controls or the fever control group. That the failure of the vaccine to induce LMI was not due to its low potency is clear from the fact that most of the

vaccinated individuals had the expected antibody response. Since no similar study in humans is available, the result can only be compared with the animal model (6, 9, 20), with which it agrees. As mentioned earlier, only the live vaccines induce CMI in mice. If the development of CMI (as indicated by LMI) can be correlated with protection in typhoid as a result of further studies, it will become necessary to revise our views on TAB vaccination (which is a killed vaccine) and its role in protection.

The observations of LMI in patients of typhoid revealed some interesting facts. Firstly, a significantly higher percentage of individuals (68%) suffering from typhoid show a positive LMI when compared with other groups. Secondly, the degree of inhibition in LMI was found to be much less than that reported in other infections (3, 10, 11). It should be noted that only the typhoid patients who were sick enough to be hospitalized were included in this study. Therefore, the actual percentage and degree of inhibition in LMI in patients with typhoid may actually be more than that observed in the present work. All the cases of typhoid fever with negative LMI were either in the first few days of illness or had some complicating factor (Fig. 6). These preliminary data, however, are not sufficient to evaluate the role of CMI in protection against typhoid fever. Further studies to elucidate this point are in progress.

It is apparent that the development of LMI and humoral responses are independent of each other even though, in most cases of typhoid fever, the two develop simultaneously. The humoral responses appear during typhoid fever as well as after TAB vaccination, but LMI develops only in response to infection by live organisms and not after immunization with killed TAB vaccine.

ACKNOWLEDGMENT

We are thankful to J. L. Turk for useful suggestions. The technical assistance of R. L. Taneja and Vasanti Rao is gratefully acknowledged. Thanks are also due to Shantha Madhavan, Statistical Officer, Indian Council of Medical Research, New Delhi, for analyzing the data.

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