IMMUNOLOGICAL STUDY OF TYPHOID FEVER IN MAN
II. CELL-MEDIATED IMMUNE RESPONSE

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INTRODUCTION

The nature of protective immunity or the mechanism of acquired resistance against enteric fever in man is not well understood. In the animal model of typhoid fever, it has been suggested that cellular immunity plays an important role in recovery from infection (Ushiba, 1965; Blanden *et al.*, 1966). In man it was also demonstrated that cell-mediated immunity developed in patients suffering from acute typhoid fever as well as in those recovering from typhoid fever but little is known about it precise role in protection (Kumar *et al.*, 1974). A recent study in man indicated that clinical recovery of complicated cases coincided with development of cell-mediated immune response (Balakrishna *et. al.*, 1977). It could be interpreted to suggest that cell-mediated immunity plays a protective role in typhoid fever.

Several methods for studying cell-mediated immune response have been developed such as leukocyte migration inhibition technique in capillary tube (Soborg and Bendixen, 1967) or in agarose gel (Clausen, 1971). However, the technique used in man is still time consuming, requiring large number of leukocytes. A simple, specific, reproducible and sensitive *in vitro* method is needed to measure cellular immunity. The leukocyte adherence inhibition test, developed by Halliday and Miller in 1972 has been used as a simple test for detection of cell-mediated immunity and serum blocking factors in patients with cancer. This assay appears to be simple, and inexpensive; it was then selected to study cell-mediated immunity in typhoid fever and to identify the antigen responsible for this reaction.

MATERIALS AND METHODS

Bacterial strains: *Salmonella typhi* 0901 was used.

Subjects: The study was conducted in 29 patients with typhoid fever whose blood were either positive by hemoculture (22 patients) or had high agglutinin titer ($\geq$ 1:320;7 patients). In addition 4 patients with paratyphoid fever were studied. The control included 18 healthy individuals, and 14 patients with pyrexia of unknown origin.

Preparation of antigen: The lipopolysaccharide (LPS) was extracted twice in 45% phenol according to the method of Neoh and Rowley (1970). The Barber protein (CB) was prepared as described by Barber *et al.*, (1966).

Widal test: The classical tube method of Widal O agglutination was used (Bailey and Scott, 1974). The optical density of the bacterial suspension was adjusted to give 0.21 at 650 nm as measured by Coleman Junior II spectrophotometer which was equivalent to 9×10^8 CFU per ml. The antigen was found to give the same titer as the Widal O antigen from Difco (Batch no. 2842-56, control 623285) when tested against the same sera.

Indirect hemagglutination (IHA) test: The LPS used to sensitize sheep red blood cell (SRBC) was prepared from *S. typhi* 0901 according to the technique of Neoh and Rowley (1970). The LPS was rendered

alkaline by treatment with NaOH according to the technique of Auzins (1968). The microtiter technique of indirect haemagglutination modified from the macrotechnique originally described by Auzins (1968) was used.

The leukocyte adherence inhibition test (LAI): Peripheral blood leukocytes from patients were prepared from heparinized venous blood sedimented with dextran solution as described by Clausen (1973) except the concentration of dextran solution and ratio of dextran solution and blood were different. The dextran solution was 3.5% and the mixture of blood and dextran was 2:1 The final cell concentration was adjusted to $2x10^7$ cell/ml. The LAI test was carried out according to the method of Halliday *et al.*, (1974), in the presence of 25 µg of Barber protein (CB) or 25 µg of twice extract lipopolysaccharide (TE-LPS) in 0.2 ml of cell suspension. The result was expressed as LAI index which was determined by the formula:

$$\text{LAI index} = \frac{\text{mean number of cells adhering in the presence of antigen}}{\text{mean number of cells adhering in the absence of antigen}}$$

Agarose gel electrophoresis: The method used was essentially that of Johansson (1972).

RESULTS

The antigen were tested whether they could cause nonspecific LAI reaction with leukocytes from healthy individuals, patients with febrile illness unrelated to typhoid fever and patients with paratyphoid fever. Mean LAI index in patients with typhoid fever, patients with paratyphoid fever, patients with PUO and healthy controls in the presence of CB and TE-LPS is shown in Table 1 and Fig. 1. Statistical analysis by 't' test showed

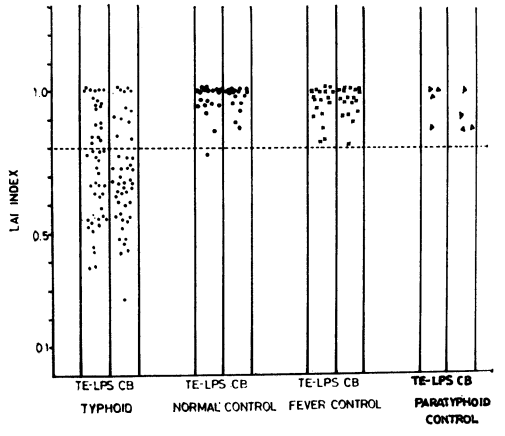

Fig. 1—The leukocyte adherence inhibition index as determined by LAI test in patients with typhoid fever, pyrexia, paratyphoid fever and healthy controls.

Table 1

LAI test in patients with typhoid fever, pyrexia of unknown origin, paratyphoid and healthy controls.

Patients	No. tested	Mean LAI ± SE in the presence of	
		25 µg Barber protein	25µg TE-LPS
Typhoid fever	29	0.71±0.03	0.75±0.03
PUO	14	0.99±0.02	0.91±0.02
Paratyphoid fever	4	0.92±0.05	0.98±0.04
Healthy controls	18	1.00±0.02	0.99±0.02

Table 2

The LAI test in typhoid patients and controls.

LAI reaction	Percent positive LAI in subjects		
	Typhoid (29)*	Healthy control (18)	PUO control (14)
Positive with both antigens	59.6	none	none
Positive with LPS negative CB	2.13	5.56	none
Negative LPS positive CB	14.9	none	none
Negative with both antigens	23.4	92.71	100

*Number of cases investigated.

that the mean LAI index in patients with typhoid fever is significantly lower than those in patients with PUO ($p < 0.01$) and in healthy controls ($p < 0.01$). Analysis using Kruskall-Wallis one way analysis of variance showed no significant difference between LAI indexes in patients with PUO patients with paratyphoid fever and healthy controls ($p > 0.05$). It was found that almost all patients with PUO, patients with paratyphoid fever and healthy controls had LAI index higher than 0.80. This figure was then chosen for distinguishing between positive and negative LAI reaction. Based on this criteria, it was found that 77% of patients with typhoid fever were positive against both types of antigens (Table 2). Only one of 18 healthy controls (5.6 %) was positive against TE-LPS but none of them was positive against CB. All 4 patients with paratyphoid fever were negative.

Time course study on positive LAI reaction in patients with typhoid fever showed that positive reaction was apparent even during the first week of illness and remained positive thereafter (Fig. 2). From the second week onwards, there was no statistical significant changes in LAI activity as analysed by Fisher exact probability test ($p > 0.05$). In contrast, there was a steady rise in the Widal O agglutinin and HA titers during the four weeks of study (Table 3).

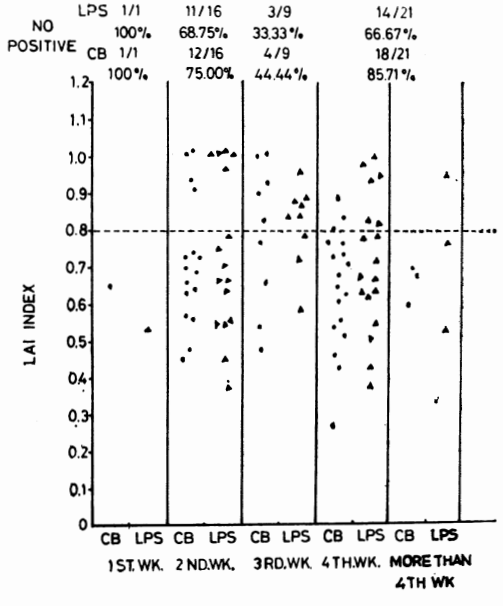

Fig. 2—LAI index at different stages of typhoid fever.

Table 3

The antibodies and LAI indices at different stages of typhoid fever.

	Duration of illness			
	1st wk. (1)*	2nd wk. (16)	3rd wk. (9)	4th wk. or more (21)
Mean O titer**	10	128.84	253.98	416.70
Mean HA titer**	320	320	806.35	730.33
Mean LAI index ± SE				
TE-LPS	0.54±0	0.75±0.24	0.82±0.11	0.72±0.19
CB	0.65±0	0.73±0.20	0.80±0.21	0.66±0.17

*Number of cases investigated.
**Reciprocal geometric mean.

The suitability of TE-LPS and CB for use in the LAI test at the same concentration of 25 μg per test was assessed by comparison of the 2 slopes of the regression lines shown in Fig 3. 't' test analysis showed that there was no significant difference between these two slopes ($p>0.8$).

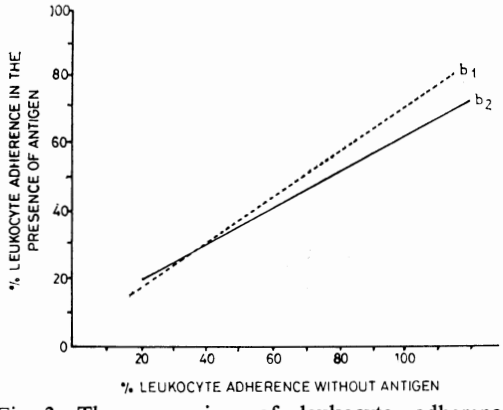

Fig. 3—The regression of leukocyte adherence inhibition in the presence of LPS (. . . .) and CB (——) antigens.

The crude Barber protein purified by agarose electrophoresis consisted of 3 main peaks designated F_1, F_2 and F_3 (Fig. 4). Each fraction was pooled, dialysed against distilled water at 4°C and lyophilised. At

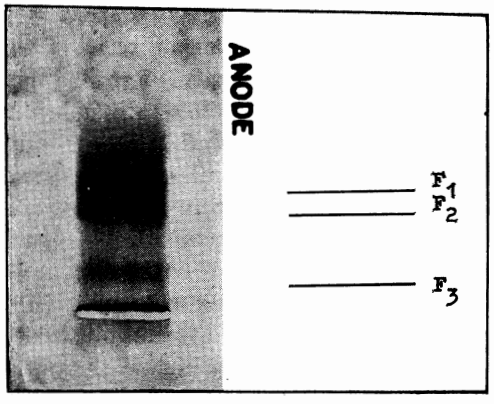

Fig. 4—Separation of protein antigen (10 mg/ml) on agarose gel electrophoresis.

the dosage of 25 μg per test, it was found that each fraction exhibited LAI activity against leukocytes from 10 patients with typhoid fever, but not against leukocytes from 3 healthy controls (Fig. 5). The LAI reaction when fraction 2 was used was significantly different from those in which the other two fractions were employed as analysed by Friedman two way analysis of variance ($p<0.0005$).

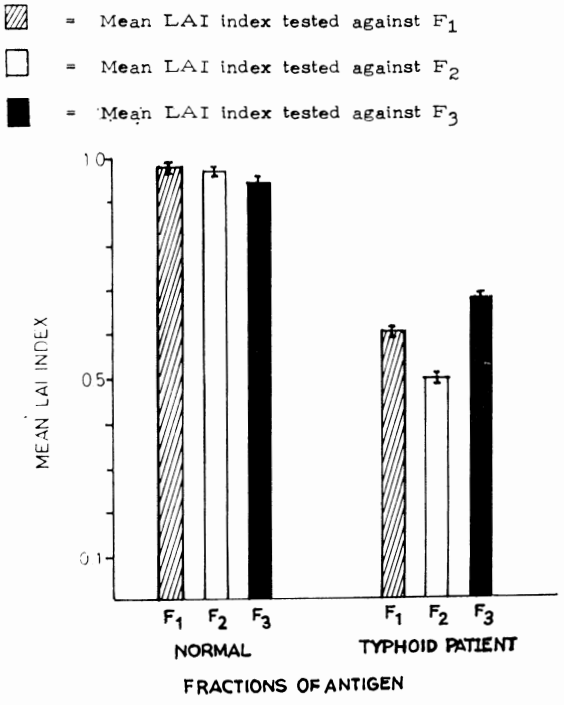

Fig. 5—The mean LAI index of typhoid patients and controls tested against fraction 1,2 and 3 of partially purified Barber protein antigen.

DISCUSSION

The study reported here employed LAI test to detect the cell-mediated immunity against typhoid fever in man. Since both LPS and CB antigens could be used in the test, it should be of interest to know which antigen was more suitable for use in this test. The result showed that there was no statistical difference in term of suitability between both antigens in the test. However, the false positive reaction was evident only when LPS was used and not when the CB antigen was employed indicating that CB may be better than LPS in the LAI test as far as the specificity of the test was concerned. However, CB is recommended for use in future studies for following reasons. Firstly, no false positive LAI reaction was observed in healthy individuals in the presence of CB. When LPS was present, 5.6% of the healthy control was positive. This non-specific LAI activity may be due to the low proliferative response of B lymphocyte to endotoxin (Ivanyi and Lehner, 1974). Another reason for false positive LAI activity was contamination of LPS by protein. This possibility was thought unlikely because the protein cotamination in our preparation was only 0.14%, which is by far too small to be responsible for the observed inhibition of adherence. Secondly, being protein in nature, the CB could be further purified to obtain components which could elicit better LAI reaction.

Our result showed that 77% of patients with typhoid fever developed CMI following a natural course of disease. This result was comparable to those of Kumer *et al.*, (1974) and Balakrishna *et al.*, (1977) who reported that CMI was noted in 68% of 22 cases and 66% of 60 cases respectively. It was observed by Kumar *et al.*, (1974) that as high as 40% of healthy controls and 31% of 16 patients with pyrexia non-related to fever were positive indicating a high degree of false positivity. In contrast, only 1 out of 18 healthy controls in the present study was positive when LPS was used. The reason for this descrepancy is unknown, Kumar *et al*,, (1974) maintained that people in the endemic area are constantly exposed to sub-infective dose of *S. typhi*, and thus would represent a degree of basal CMI in the population with concomitant resistance to *S. typhi* infection. Another explanation of non-specific CMI reaction in Kumar's study was the non-specific stimulation of B lymphocytes by endotoxin (Ivanyi and Lehner 1974; Miller *et al.*, 1978) which may trigger B cells to produce MIF (Rocklin *et al.*, 1974). Alternatively the antigen used by Kumar was a very crude ultrasonic disrupted cell suspension which may contain antigens shared by other *Salmonella*. Such enterobacterial common antigen (Makela and Mayer, 1976) devoid of

LPS activity could be responsible for such non-specific CMI reaction in healthy individuals.

Failure to demonstrate gradual rise in CMI activity throughout the course of illness in the present study is not clear. Whether antimicrobial therapy has suppresive activity on CMI response in patients with typhoid fever remains to be investigated.

SUMMARY

The development of cell-mediated immune response to lipopolysaccharide and Barber protein from *Salmonella typhi* was investigated in patients suffering from typhoid fever. The cell-mediated immunity as measured by the leukocyte adherenc inhibition test, was demonstrable in 77% of patients with typhoid fever but only in 5.6% of healthy controls. It was found that cell-mediated immune response appeared after the first week of illness and persisted for at least 4 weeks. The time course development of cell-mediated immune response and humoral immune response was correlated but the magnitude of each response was independent of one another.

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