J Diarrhoeal Dis Res 1994 Jun;12(2):97-102
0253-8768/94 $ 1.00+0.10

Peripheral Blood Granulocytes and Mononuclear Cell Responses in Monkeys with Experimental Shigellosis

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ABSTRACT

Changes in neutrophil response to N-formyl-methionyl-leucyl-phenylalanine (FMLP) and the phenotype of peripheral blood mononuclear cells were studied in monkeys after oral challenge with *Shigellae*. Monkeys were first challenged with *S. dysenteriae* 1 which caused shigellosis in some of the monkeys. After recovery, the monkeys were rechallenged with *S. flexneri* 2a. No difference in sensitivity was observed in the monkeys during shigellosis caused by either *S. dysenteriae* 1 or *S. flexneri* 2a. The optimal dose of FMLP for neutrophil polarization, a measure of early cell activation, in normal healthy monkeys was 10^{-7} M when 67% of the neutrophils were polarized. Neutrophils from monkeys ill with shigellosis required higher doses of FMLP (10^{-6} and $5X10^{-7}$ M) for maximum polarization. As the monkeys recovered, a gradual decrease in the doses of FMLP for optimal neutrophil polarization was also observed. The percentage of CD2-positive T lymphocytes, the earliest marker for T lymphocytes in the peripheral blood, decreased when the monkeys developed shigellosis and returned to normal levels as the monkeys improved. However, there was no change in the percentage of CD20-positive peripheral blood B lymphocytes.

Key words: Neutrophil polarization; Lymphocyte phenotype; Monkeys; Shigellosis.

INTRODUCTION

The immune response in shigellosis has not been clearly elucidated. Studies on humans carried out so far have concentrated on specific antibodies to various antigens of *Shigella*. Thus, antibodies to the lipopolysaccharide (LPS), invasion plasmid antigens, and Shiga toxin have been found in serum, saliva, stool, breast-milk, and duodenal aspirate (1-7). Furthermore, antibody secreting cells to the LPS of *S. dysenteriae* 1 and *S. flexneri* have been demonstrated in infected individuals (8). Very few studies have addressed the question of cellular immunity in shigellosis. Natural killer cells have been shown to exert cytotoxicity against *S. flexneri* infected cells (9,10) and a CD4+T lymphocyte clone directed against *S. flexneri* has been generated from an infected individual (11). Locally, there is an increase in the percentage of CD8+T lymphocytes in the lamina propria and in intra- epithelial lymphocytes (IELs) as well as in the expression of major histocompatibility antigens II (MHCII) by IELs (12).

Lack of a suitable animal model for shigellosis makes studies on its immunopathogenesis difficult. Monkeys are commonly used although the infectious dose is high. Studies conducted on monkeys, which were done mainly to assess vaccine efficacy, have again concentrated on antibody responses (13). None of these studies assessed cellular immunity. In this study we have attempted to address some aspects of cellular immunity by describing changes in neutrophil polarization in response to N-formyl-methionyl-leucyl-phenylalanine (FMLP) and lymphocyte phenotype in monkeys following challenge with different species of *Shigellae*.

MATERIALS AND METHODS

Nineteen healthy monkeys were obtained from Mirpur zoo, Dhaka, Bangladesh and were kept in quarantine for 4 weeks before bacterial challenge.

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Preparation of *Shigella* for oral challenge. The strains of *S. dysenteriae* 1 and *S. flexneri* 2a used in this study were isolated from patients at ICDDR,B. These are characterized strains and are part of the ICDDR,B culture collection. They were subcultured on trypticase soy agar and grown in Casamino Acids-yeast extract broth medium (14) for 22 hrs at 37°C with shaking. Bacteria were centrifuged at 6,000xg, washed with physiological saline, adjusted to 0.6 OD at 600 nm (10^9CFU/ml).

Oral challenge of monkeys with *Shigella* species. Monkeys were kept in individual cages and sedated prior to challenge. Bacteria in 10 ml of physiological saline were fed through a nasogastric tube. Fifteen monkeys were challenged with *S. dysenteriae* 1 by feeding $5x10^{10}$-$1x10^{11}$ bacteria per animal. After recovery, 10 of the above monkeys were rechallenged with *S. flexneri* 2a at a dose of $5x10^{11}$ bacteria per animal. Four monkeys were not challenged and served as controls. Furthermore, as samples were also collected prior to challenge from each monkey, each monkey acted as its own control.

Clinical monitoring of monkeys. Only healthy monkeys and monkeys whose stools were free of *Shigella* on culture were used in the study. After challenge, the monkeys were monitored daily for their general appearance, appetite, food intake, urine output, rectal temperature, and stool appearance and output. Monkeys were classified as having mild to moderate or severe illness depending on the severity of diarrhoea/dysentery. In mild illness, the stool was loose and contained mucus with no obvious blood, whereas in moderate illness a small amount of blood was always present. Severe illness was characterized by loose stools with mucus and frank blood (Table I).

Table I. Clincal observations in monkeys after bacterial challenge.

No. of monkeys challenged	Challenge bacteria	Post-challenge observations in monkeys			
		No. of monkeys without illness	No. of monkeys with mild illness*	No. of monkeys with moderate illness**	No. of monkeys with severe illness***
15	*S. dysenteriae* 1	6	3	3	3
10	*S. flexneri* 2a	5	2	0	3
4	None	4	0	0	0

* mild illness = monkeys with loose stools containing mucus without obvious blood
** moderate illness = monkeys with loose stools containing mucus and some blood
*** severe illness = monkeys with loose tarry stools containing mucus and frank blood

Isolation of peripheral blood mononuclear cells and granulocytes. Venous blood was collected aseptically into heparinized containers and layered on Ficoll-hypaque (Pharmacia). After centrifugation at 500xg for 25 minutes, peripheral blood mononuclear cells (PBMs) were collected from the interface of plasma and Ficoll-hypaque, and washed twice in Minimal Essential Medium (MEM) (Gibco) with 2mM HEPES, 2% foetal bovine serum (FBS) (Gibco), 2mM glutamine, 50 IU/ml penicillin and 50 g/ml streptomycin (Flow). Cells were counted by the trypan blue exclusion technique before use. Granulocytes, which formed a pellet with red blood cells (RBC), were isolated by sedimentation through dextran (Sigma) after which they were washed twice using a mixture of Hank's balanced salt solution (HBSS) and 3-(N-morpholino) propanesulphonic acid (MOPS) at 10mM. Remaining RBC were lysed by hypotonic shock. Granulocytes were finally resuspended at 10^6 cells/ml and counted. In all cases, the cell suspension consisted of more than 95% neutrophils with the remainder being mainly basophils and eosinophils.

Polarization assay for neutrophils. The polarization or shape change assay for neutrophils was carried out against various concentrations of the pure chemotactic factor FMLP (Sigma). Neutrophils at 10^6/ml in HBSS-MOPS were incubated with log dilutions of FMLP ranging from 10^{-5} M to 10^{-10} M for 30 min at 37°C. Cells were then fixed with 2% glutaraldehyde in HBSS-MOPS, washed twice, and finally resuspended in a few drops of HBSS-MOPS. The percentage of polarizing cells was assessed by viewing under a light microscope (Olympus BH-2) using a 40x objective. Neutrophils with spherical outlines or with a few filamentous projections were counted as round cells. Any cell deviating from this spherical morphology, including cells with a constriction separating a spherical outline of the membrane from a ruffled outline, was scored as a polarized cell (16). Polarized cells were expressed as a percentage of the total granulocytes counted.

Determination of surface phenotype of peripheral blood mononuclear cells. PBMs were phenotyped by indirect immunoflourescence for CD20 using the monoclonal antibody (MAB) B1 at 1:10 (Coulter Immunology, USA). A second layer of flourescein-conjugated anti-mouse immunoglobulin (Dakopatts) was used at 1:40. The percentage of cells staining for the MAB was determined by counting under a flourescence microscope when at least 100 cells were counted. PBMs were also phenotyped for CD2 by E rosetting with 4% 2-aminoethyl isothiouronium bromide hydrobromide (AET) (Sigma)- treated sheep red cells (SRBC) (15). Briefly, PBMs at 10^7/ml were mixed with equal volumes of AET-SRBC and FBS for 1hr at 37°C. The mixture was gently resuspended, a

drop taken on a glass slide and and the percentage of rosetting cells (any cell with 3 or more RBC around it) was determined by counting under a light microscope (at least 100 cells were counted).

Statistical analysis. Student's *t* test was used to compare lymphocyte phenotype of healthy versus ill monkeys on different days.

RESULTS

Clinical observations in monkeys following challenge with *Shigella*. Following challenge with *S. dysenteriae* 1, 6/15 monkeys did not develop any illness, 3/15 had mild diarrhoea, 3/15 were moderately ill and 3/15 had a severe illness (Table I). After the challenge with *S. flexneri* 2a, 5/10 monkeys did not develop any illness, 2/10 had mild illness and 3/10 developed a severe illness (Table I). In most monkeys who were ill, clinical symptoms of shigellosis appeared within 2-3 days. However, some monkeys were ill as early as 8-12 h after challenge. Along with the passage of bloody, mucoid stools monkeys were listless, with lowered food intake and reduced urine output. The rectal temperature in some monkeys was elevated.

Neutrophil polarization in healthy monkeys. Neutrophils from healthy monkeys suspended in HBSS-MOPS without a chemotactic factor remained spherical after incubation for 30 min at 37°C (Fig. 1A). In the presence of different concentrations of the chemotactic factor FMLP, neutrophils became polarized (Fig. 1B). However, there were differences in the proportion of polarized cells as well as in the appearance of these neutrophils at different FMLP concentrations. Maximum polarization was observed at 10^{-7} M FMLP when 67% of the neutrophils were polarized. At high FMLP concentrations, such as 10^{-5} or $5X10^{-6}$ M, the extent of polarization was less, and polarized cells showed an irregular outline due to the formation of contraction waves generating from all points of the cell surface. At lower FMLP concentrations (10^{-10} M), some neutrophils were well polarized and elongated although the percentage of neutrophils polarizing decreased to 20. The results of 7 experiments using neutrophils from 7 healthy monkeys are shown in Fig. 2.

Neutrophil polarization in monkeys with mild to moderate shigellosis. Following oral challenge, neutrophils from 7 monkeys with mild to moderate illness were maximally polarized on the average at 10^{-6} M FMLP (Fig. 2). It was not possible to differentiate between mild and moderate illness on the basis of neutrophil polarization as there was considerable overlap in the percentages of neutrophils polarizing between the concentrations $5x10^{-7}$ and 10^{-6} M FMLP when monkeys were mildly or moderately ill. However, neutrophils from monkeys who showed no signs of illness following challenge were maximally polarized at 10^{-7} M FMLP (Fig. 2).

Neutrophil polarization in monkeys with severe shigellosis. Neutrophil polarization was examined in 4/6 of the severely ill monkeys (Table II). Neutrophils from these monkeys showed maximum polarization at $5X10^{-6}$ M FMLP during the acute illness. Interestingly, as the monkeys recovered from the illness, lower concentrations of FMLP were required to obtain maximum neutrophil polarization. The time required for recovery varied from one monkey to another, and in one of the very ill monkeys this was as long as 3 months. The maximum effective concentration of FMLP for neutrophil polarization in this monkey was 10^{-7} M prior to challenge. After challenge, the maximum effective concentrations were: 10^{-6} M on the 2nd and 6th days when the monkey was moderately ill, $5x10^{-6}$ M on the 13th and 20th days when severely ill, $5x10^{-7}$ M on the 67th day when much improved, and 10^{-7}

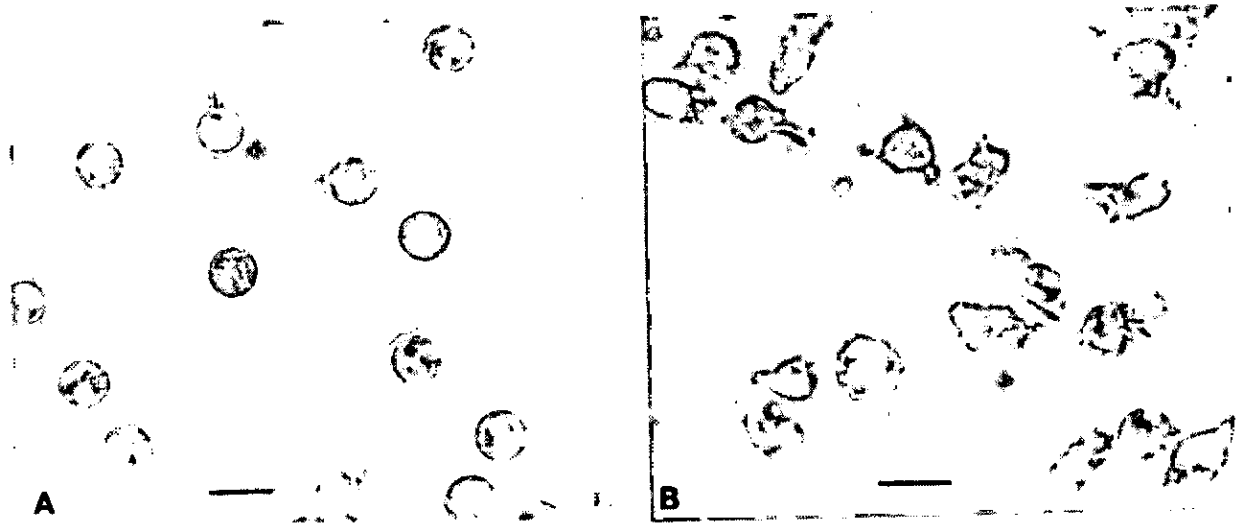

Fig. 1. Photomicrographs of monkey neutrophils fixed in suspension after incubation for 30 min at 37°C in (A) HBSS-MOPS and (B) 10^{-7} M FMLP in HBSS-MOPS. Bar = 10μm.

M on the 128th day when completely well (Fig. 3; data of days 6, 13 and 128 are not shown for the sake of simplicity).

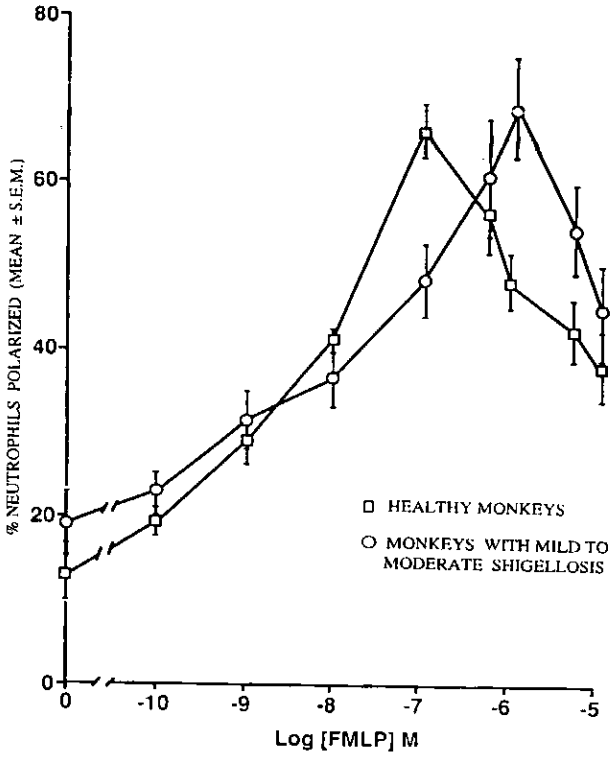

Fig. 2. Dose response of FMLP for neutrophil polarization in healthy monkeys and in monkeys with mild to moderate shigellosis. The abscissa shows the dose of FMLP and the ordinate shows the mean percentage of neutrophils polarizing ± standard error of the mean (SEM).

Table II. Dose response of neutrophil polarization in FMLP in the severely ill monkeys before and after challenge with *Shigellae*.

Dose of FMLP	Percentage of neutrophils polarized (mean±SEM)			
	day 0	day 2	day 20	day 67
0	15.5±4.5	24.0±10.0	17.0±2.0	13.5±3.5
10^{-10}M	22.0±1.0	30.5±7.6	ND	ND
10^{-9}M	28.0±2.0	34.5±9.6	29.5±5.6	42.5±2.5
10^{-8}M	40.0±3.0	40.0±10.0	35.5±6.6	45.5±0.5
10^{-7}M	68.0±6.0	49.5±8.6	43.5±6.6	65.0±6.1
$5x10^{-7}$M	65.0±1.0	57.0±4.1	50.0±6.1	70.0±1.0
10^{-6}M	58.0±8.0	65.5±15.6	54.5±8.6	62.0±2.0
$5x10^{-6}$M	54.0±8.0	59.0±17.1	65.0±7.1	59.5±0.5
10^{-5}M	49.5±8.5	51.0±16.1	44.5±1.5	54.5±5.6

ND = not done

Surface phenotype of monkey PBMs. Prior to challenge with *S. dysenteriae* 1, 54.5% (range 40-72%) of PBMs from 8 monkeys were CD2-positive T lymphocytes (Fig. 4A). The percentage of peripheral blood CD2-positive T lymphocytes decreased to 35.7 (range=28-41%; data from 3 monkeys) (p=0.015) on the 2nd day following challenge and to 47.1 (range =23-59%) (p=0.18) on the 6th day (tested on all 8 monkeys). The percentage of CD2-positive T lymphocytes returned to normal 13 days after challenge as shown in 6 monkeys (51.5%, p=0.5) (Fig.4A). When each monkey was followed individually, 2/8 showed marked decrease in the percentage of CD2-positive T lymphocytes, one 2 days after challenge and the other 6 days after challenge.

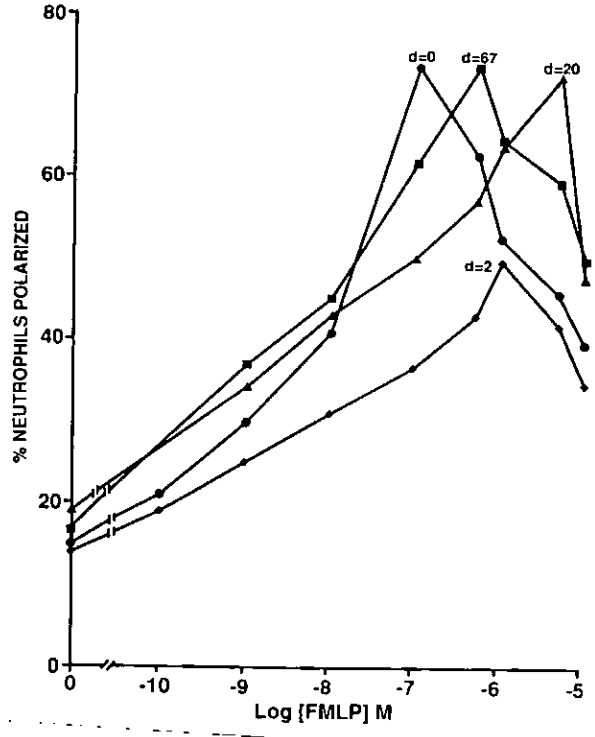

Fig. 3. Dose response of neutrophil polarization in FMLP for one monkey before and after challenge with *S. dysenteriae* 1. The abscissa shows the dose of FMLP and the ordinate shows the percentage of neutrophils polarizing. Data is shown for d=0 (day 0) i.e. before challenge, and days 2, 20 and 67 after challenge.

Prior to challenge with *S. flexneri* 2a, 75.4% of PBMs from 5 monkeys were positive for CD2 (Fig.4B) and 11.5% of PBMs from 4 monkeys were positive for CD20. Three days after challenge, no change was observed in the percentage of peripheral blood CD2-positive T lymphocytes. However, 8 days after challenge the percentage decreased to 55.2 (range=22-69%) (p= 0.05) with a marked decrease observed in one of the monkeys (Fig.4B). The percentage of peripheral blood CD20-positive B lymphocytes remained unchanged 3 (8.3%, p=0.2) and 8 (15%, p=0.5) days after challenge with *S. flexneri* 2a.

DISCUSSION

A variable number of monkeys became ill after challenge with *S. dysenteriae* 1 and *S. flexneri* 2a and there was also a variation in the extent of illness. However, monkeys challenged with *S. dysenteriae* 1 were generally ill sooner after challenge than those challenged with *S. flexneri* 2a. For this reason, blood was collected at earlier time points from monkeys challenged with *S. dysenteriae* 1 than from monkeys challenged with *S. flexneri* 2a.

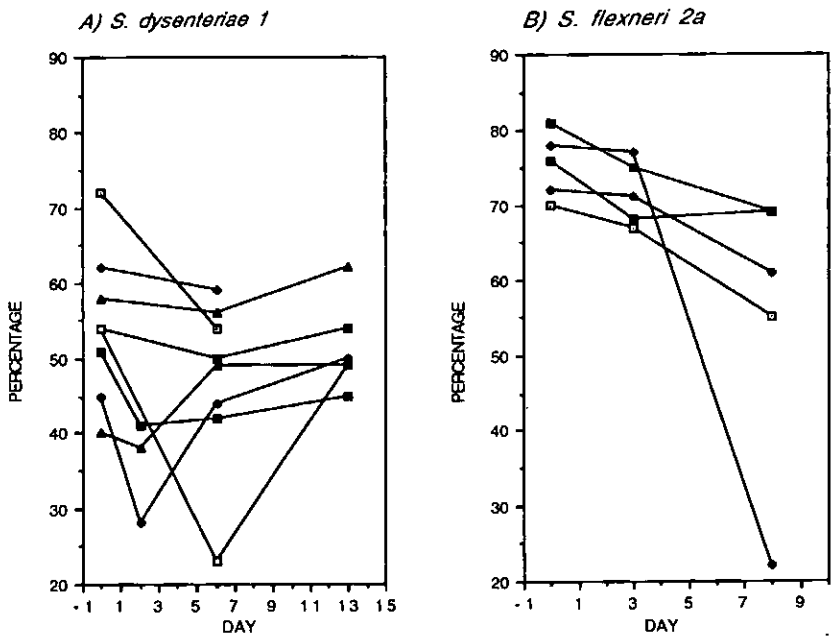

Fig. 4. Percentage of CD2 positive T lymphocytes in the peripheral blood of monkeys before (do) and after challenge with A) *S. dysenteriae* 1 and B) *S. flexneri* 2a. The abscissa shows days before and after challenge and the ordinate shows the percentage of CD2 positive T lymphocytes. Each symbol indictaes an individual monkey.

We have used the polarization or shape change assay to study changes in monkey neutrophil response to the chemotactic factor FMLP on several days following oral challenge with *S. dysenteriae* 1 and *S. flexneri* 2a. As these assays were done in suspension, there was no interaction between the cells and the substratum, and any alteration in the morphology of the cells is therefore directly related to the presence of the chemotactic factor, i.e. FMLP. Polarization is an initial event in locomotion and can be scored accurately as has been shown in neutrophils, monocytes and lymphocytes (16-19). This report shows, for the first time, that the optimal dose of FMLP inducing neutrophil polarization in normal monkeys is 10^{-7} M when 67% of the neutrophils polarize. The optimal dose of FMLP for polarization of human neutrophils is 10^{-8} M which induces polarization in more than 90% of the neutrophils (16). This variation in the optimum FMLP dose could be due to the presence of fewer receptors or difference in receptor sites for FMLP on the surface of monkey neutrophils compared to those of human neutrophils. In shigellosis, whether induced by *S. dysenteriae* 1 or *S. flexneri* 2a, the ability of neutrophils to polarize in response to FMLP is reduced since neutrophils from ill monkeys, particularly severely ill monkeys, required higher doses of FMLP for optimal polarization. However, this reduction is reversible as on recovery the dose of FMLP for optimal neutrophil polarization approached normal levels. This alteration in neutrophil polarization could either be due to a direct effect of *Shigella* itself or be a consequence of acute infection where there is a rapid outpouring of neutrophils from the bone marrow. So far there is no evidence to support the former hypothesis. However, it is known that shigellosis may be accompanied with leukaemoid reaction in humans where the total white-cell count exceeds 40,000/cmm of blood. The leucocytosis is mainly due to immature granulocytes (20,21). It is possible that a similar leucocytosis also occurs in monkeys ill with shigellosis.

Alteration in the phenotype of gut lymphocytes has been reported in adults with shigellosis (12) as well as in that of peripheral blood lymphocytes of children with shigellosis with leukaemoid reaction (22). Thus, in the gut there is increased expression of MHCII antigens as well as increases in the percentages of CD4-positive and CD8-positive T lymphocytes (12). However, it is not known whether this is accompanied by similar changes in the peripheral blood. In children there is a decrease in the percentage of CD2-positive T lymphocytes and a corresponding increase in null cells in the peripheral blood when shigellosis is accompanied with leukaemoid reaction (22). These authors suggest that T lymphocyte defect contributes to the severity of illness. We have also observed reduced percentages of CD2- positive T lymphocytes in the peripheral blood of some of the monkeys ill with shigellosis. Severe reduction was observed in 2 monkeys after challenge with *S. dysenteriae* 1, one of whom was severely ill, and the other had a mild illness. Of the 5 monkeys whose PBMs were phenotyped following challenge with *S. flexneri* 2a, only one monkey was severely ill and that monkey had a marked reduction in the percentage of CD2-positive T lymphocytes (Fig. 4). Unfortunately, the number of monkeys studied here is small so that it is difficult to ascertain whether decrease in the percentage of CD2- positive T lymphocytes is related to the severity of illness.

These findings observed on a small number of monkeys, suggest that some aspects of cellular immunity

may change in shigellosis which may be relevant to disease outcome.

ACKNOWLEDGMENTS

This research was supported by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) where most of this work was carried out under a collaborative research programme with Dhaka University. The ICDDR,B is supported by countries and agencies which share its concern for the health problems of developing countries. Current donors include: the aid agencies of the governments of Australia, Bangladesh, Belgium, Canada, Denmark, Japan, the Netherlands, Norway, Saudi Arabia, Sweden, Switzerland, the United Kingdom and the United States; international organizations including the United Nations Children's Fund, the United Nations Population Fund and the World Health Organisation; and private foundations, including the Ford Foundation and the Sasakawa Foundation.

We thank the staff of the animal house of ICDDR,B for taking care of the monkeys.

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