SHIGELLOSIS IN CYROMOLGUS MONKEYS (MACACA IRUS)

IV. BACTERIOLOGICAL AND HISTOPATHOLOGICAL OBSERVATIONS ON THE EARLIER STAGE OF EXPERIMENTAL INFECTION WITH SHIGELLA FLEXNERI 2A

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The locality of multiplication of S. flexneri 2a in the alimentary tract and the causal relation between the distribution of the fluorescing bacilli in the tissue and the appearance of inflammatory response were investigated on 4 cynomolgus monkeys in the earlier stage of 12 and 24 hr after an oral administration of the bacilli.

The results of the present observations suggested the importance of the involvement of epithelial cells, for the multiplication of the bacilli. In the small intestine, there was evidence neither of penetration of the bacilli into the epithelial cells nor of a pronounced increase in the number of the bacilli in the lumen.

The degeneration of the epithelial cells had already taken place when the bacilli penetrated into the cells. This fact would suggest that some preconditioning on epithelial cells by certain cytotoxic substances seems to be necessary for the penetration of the bacilli into them.

The penetration of the bacilli into the lamina propria via the intact epithelial barrier was not observed in the earlier stage of infection; this phenomenon appeared 3 to 4 days after infection when the surface mucosa degenerated to necrosis and desquamated.

The presence of the bacilli does not necessarily coincide with the locality of the lesions.

INTRODUCTION

In order to clarify the pathogenesis of bacillary dysentery it is necessary to know the locality of multiplication of the bacilli in the alimentary tract. As regards this problem, Koya & Kosakai (1956) inferred from the observations on human beings that the multiplication of Shigella bacilli in the lumen of the small intestine must be the prerequisite step for the establishment of the infection. Formal et al. (1963) and LaBrec et al. (1964), furthermore, concluded from their study of experimental dysentery in guinea pigs and monkeys that the penetration of the bacilli into intact epithelial cells and lamina propria was the essential factor in the infection.

The purpose of the present studies is the examination of the primary site of multiplication of the bacilli to produce inflammation in the large intestine in experimental dysentery of cynomolgus monkeys, successfully carried out by Honjo et al. (1964). The studies consist of an estimation of the number of bacilli in the intestinal lumen and an
investigation of the causal relation between the penetration of bacilli into the mucous membrane and the appearance of lesions at the earlier stage after oral administration of *Shigella flexneri* 2a. The findings at the severest stage of the disease, which have already been reported (Honjo et al., 1964 and Ogawa et al., 1964), will again be analyzed here, together with the materials obtained later, in comparison with the data of earlier stage of infection.

**MATERIALS AND METHODS**

*Animals*: Animals used were eight apparently healthy cynomolgus monkeys (*Macaca irus*) weighing from 2.28 to 3.79 kg. These monkeys were imported from the Philippines and conditioned at the NIH for 43 days after their arrival. The care and management of animals were done as previously reported by Takasaka et al. (1964). Their stool specimens were examined for *Shigella* at least on three occasions during this conditioning period and negative results were always obtained. They were divided into two groups of an equal number; the first group was sacrificed 12 hr after the oral administration of *S. flexneri* 2a and the second was 24 hr following the administration.

*Shigella strain used and infection procedures*: The origin of *S. flexneri* 2a administered was the same as that of the seventh experiment previously reported by Honjo et al. (1964). The bacilli were isolated from lesions in the colon of the death case from natural *Shigella* infection, monkey No. 5503. The colon had been preserved at −20°C for about 13 months. (We conventionally call these bacilli Strain 5503.)

The same infection procedures were used as those described previously (Honjo et al., 1964). The monkeys of the first group were orally challenged with approximately $5.7 \times 10^9$ of viable organism and the animals of the second group with $5.0 \times 10^9$.

*Viable counts of S. flexneri 2a in the gastrointestinal tract*: Enumeration of the test organism was carried out on Salmonella-Shigella agar by the method of Miles et al. (1938) for the contents of five portions of the gastrointestinal tract; namely, stomach, lower part of small intestine, cecum, ascending colon and rectum.

*Histological examinations*: Histological observations on the characteristics of the lesions and the distributions of *S. flexneri* 2a in tissue were made by a combination of fluorescent antibody technique and ordinary histological method in each section as in the previous publication by Ogawa et al. (1964).

**RESULTS**

*Virulence of the Strain Used.*

The strain 5503 was confirmed to be virulent for cynomolgus monkeys when it was orally administered, that is, 3 out of 4 monkeys that were used to check up for the virulence assay developed typical bloody mucous diarrhea continuing for 4 to 7 days about 1 to 2 days after the administration.

*General Clinical Findings.*

All 4 monkeys of the first group that were autopsied 12 hr after the challenge showed no clinical signs of dysentery. In the second group in which autopsy was done 24 hr later, one (No. 6198) of 4 animals had clear clinical manifestation of bloody mucous diarrhea at the time of sacrifice. The remaining 3 monkeys of the second group showed no apparent symptom.
Counts of Shigella flexneri 2a.

Figure 1 illustrates the number of S. flexneri 2a per gram of contents in five parts of gastrointestinal of each animal.

At 12 hr Postchallenge.

**Stomach**: The number of bacilli was \(10^{5.6}\) in No. 6298 and below \(10^{3.0}\) (uncountable) in the remaining 3 cases. **Small intestine**: \(10^{8.0}\) and \(10^{6.3}\) bacilli were determined in Nos. 6300 and 6298, respectively. In the other two cases, the number was below \(10^{3.0}\). **Large intestine**: The number of bacilli enumerated here was approximately the same as that in the small intestine in Nos. 6300 and 6298. In the other two cases the number was between \(10^{4.4}\) and \(10^{6.3}\), larger than that in the small intestine.

At 24 hr Postchallenge.

**Stomach**: The number of bacilli was below \(10^{3.0}\) except in No. 6224 where a local lesion accidentally was detected. **Small intestine**: \(10^{8.5}\) in Nos. 6198 and 6224 and around \(10^{4}\) in the remaining cases were counted. **Large intestine**: The number of bacilli was the same as or larger than that in the small intestine. No. 6198, which showed clinical sign, harbored more than \(10^{8.6}\) bacilli.

In Fig. 2, the number of S. flexneri 2a per gram of contents in each portion examined is plotted at different stages after the 3rd to 4th day postchallenge.

**Stomach**: Even at the stage of the severest symptom, that is 3 to 4 days postchall-
The number of bacilli was estimated at below $10^{5.8}$. Small intestine: In 3 of 6 dysenteric monkeys, the number determined was over $10^{7.0}$ 3 to 4 days postchallenge. Thirteen to 14 days postchallenge, however, it was smaller than $10^{4.0}$ in every case which recovered from dysentery. As for asymptomatic cases, all monkeys harbored less than $10^{7.0}$ bacilli throughout the course of experiment. Large intestine: From most of the dysenteric cases, the bacilli were isolated in number of over $10^{7.0}$ 3 to 4 days postchallenge. The number dropped evidently to below $10^{5.0}$ in almost all cases in the stage of recovery, 13-14 days postchallenge. It was not observed that the number of bacilli in any of the asymptomatic cases exceeded $10^{7.0}$ during the whole period of experiment.

![Fig. 2. The number of S. flexneri 2a at different stages postchallenge.](image)

○: asymptomatic case   I: stage of peak of the disease, 3-4 days postchallenge
□: dysenteric case     II: 6-8 days post challenge
[]: stage of recovery, 13-14 days postchallenge

The Relation between the Distribution of Bacilli as Examined by Means of Fluorescent Antibody Technique and the Lesions.

The Fig. 3-A & B, show diagrams modeled through the lower small intestine and the large intestine, each representing the relation between the distribution of bacilli in the tissue and the inflammatory response, as observed in 4 cases each at 12 and 24 hr after the bacilli were given.

At 12 hr Postchallenge: In No. 6300 a degeneration, such as disappearance of microvilli, vacuolation of cytoplasm, atrophy of both cytoplasm and nucleus, was observed on the surface epithelial cells of the mucous membrane almost over the entire large intestine but not in the small intestine. The bacilli were found predominantly in the epithelial cells from the ileocecal valve through the transverse colon (Figs. 9-a, b & 10-a, b). Inflammatory response, infiltration of leucocytes and edema, was limited to the lamina propria situated below the epithelium where a relatively large number of bacilli were present. However, no bacilli were found in the lamina propria or the submucosa even in inflammatory areas. In Nos. 6311 and 6317, the surface epithelial cells in the limited areas of the large intestine degenerated (Fig. 5), the bacilli being found in a few of those cells (Fig. 8-a, b). No inflammatory response was observed. In No. 6298, neither degeneration of the epithelial cells, penetration of the bacilli into
Fig. 3. The distribution of fluorescing bacilli and lesions in the intestine of each animal. From above: 1: epithelium, 2: lamina propria, 3: muscularis mucosa, 4: submucosa, 5: muscle layer, 6: serous membrane. The oblique lines show the inflammations, and the points fluorescing bacilli. Desquamation of epithelial cells laden with the bacilli.

the cells nor inflammatory response were observed, though the number of bacilli in the lumen was slightly larger than in the case of Nos. 6311 and 6317.

At 24 hr Postchallenge: The entire large intestine of No. 6198 became reddish and slightly thick; a histological examination revealed catarrhalic colitis, that is, degeneration and desquamation of the epithelium, leucocytic infiltration and edema through the lamina propria and submucosa and dilatation of blood vessels (Figs. 6 & 7). The bacilli, however, distributed in surface epithelial cells, and could be found neither in the lamina propria nor in the submucosa. Both the density of bacilli in the epithelial cells and the intesity of the inflammation tended to increase gradually toward the upper part of the large intestine. In No. 6224 the bacilli were found in some of the locally degenerated surface epithelial cells of the large intestine and leucocytes slightly infiltrated in the lamina propria below them. As regards the other parts, neither inflammatory response nor invasion of the bacilli were observed. In Nos. 6219 and 6227, the ileocecal valves were slightly swollen and congestive, and the degeneration was observed in the surface epithelial cells, a few of which were laden with the bacilli.
DISCUSSION

The Primary Site of Multiplication of S. flexneri 2a.

In the enumeration of the S. flexneri 2a in the gastrointestinal tract there was no evidence of a pronounced increase in number of bacilli in the small intestine, particularly at an earlier stage. We found it difficult, therefore, to agree with Koya & Kosaki (1956), who insisted that the multiplication of Shigella bacilli in the lumen of the small intestine appeared to be a prerequisite step for the establishment of infection in the large intestine. The present results suggested the importance of involvement of the large intestine for the multiplication of the bacilli. Furthermore, in the case which a comparatively large number of bacilli was estimated in the content of the large intestine (10^1-10^2 times those in the other cases), there had already been a marked desquamation of surface epithelial cells of the large intestine, heavily laden with the bacilli within 24 hr after infection, and no parasitized cells of small intestine during the whole period. This fact suggests that the epithelial cells of the large intestine should primarily be regarded as the locality for the multiplication of the bacilli. Further studies should be made to determine the locality of multiplication of Shigella bacilli to evoke symptom dysentery.

The Penetration of the Bacilli into the Epithelial Cells.

As has already been pointed out by LaBrec et al. (1961, 1964), it is clear that at the earlier stage of infection of S. flexneri 2a, in the experimental Shigellosis, there occurs the penetration of the bacilli into the epithelial cells in the large intestine followed by the intracellular multiplication. According to our observation, the bacilli were found exclusively in the surface epithelial cells within 24 hr after the infection, unlike the reports made by LaBrec et al. (1964): the direct penetration into the lamina
propria and multiplication of the bacilli were not observed at this period. These were observed 3 to 4 days after the infection, when the mucous membrane degenerated to necrosis and became erosive. This fact makes it hard to consider that the bacilli pass through the intact epithelium and basement membrane, thus reaching the lamina propria directly. From our observation it is suggested that the damage of the surface mucous membrane permits the bacilli to penetrate into the lamina propria. In the cases when the bacilli were found in the epithelial cells, degeneration was observed not only among parasitized cells but also among non-parasitized cells which were present in a comparatively large area around the former cells; this indicates that the degeneration had preceded before the bacilli penetrated into the epithelial cells. In other words, some pre-conditioning by certain cytotoxic substances seems to be necessary for the penetration of the bacilli into the cells. This surmise has further been confirmed by a cell culture study as reported elsewhere (Ogawa et al. in preparation). Whether this substance originates from the Shigella bacilli given or from some other source is an important question which should be explored in the future.

Causal Relation between the Presence of the Fluorescing Bacilli and the Appearance of the Inflammatory Response.

As for the relation between the presence of bacilli and the appearance of the inflammatory response, it is hard to make a conclusive statement from the present morphological study. At the early stage within 24 hr, the bacilli were exclusively found in the surface epithelial cells: even at the peak which comes 3-4 days later, the bacilli were found only in a limited areas, as deep as the lamina propria where the mucosa degenerated to necrosis and desquamated; they were not found in the submucosa. The inflammatory response, on the other hand, was sometime observed in the submucosa after 24 hr, spreading extensively. Thus in Shigellosis, the presence of the bacilli do not necessarily seem to agree with the locality of lesions contrary to the general rule in most cases such as pyogenic and granuloma-forming organisms. It is, therefore, difficult to recognize a causal relation between the appearance of the inflammatory response extended to the submucous layer and the multiplication of the bacilli in the epithelial cells. It may be necessary, as Watkins (1960) suggested, to consider the role of a hypersensitivity reaction in the pathogenesis of Shigellosis in monkeys. Further research should be made on causative elements to produce the inflammation and their pathogenic significance.

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Fig. 4 : Normal surface epithelium of large intestine. Orig. mag. ×600.
Fig. 5 : Slightly degenerated surface epithelial cells (upper half) of large intestine in No. 6317, 
12 hr postchallenge. Note the loss of striated borders, picnotic nuclei as compared with 
lower half and Fig. 4. Orig. mag. ×600.
Fig. 6 : No. 6198, 24 hr postchallenge. Catarrhal colitis. Submucous layer thickened due to 
edema and was infiltrated with leucocytes. Orig. Mag. ×50.
Fig. 7 : The same case as Fig. 6. Surface epithelial cells markedly degenerated and desquamated.
Lamina propria was infiltrated with leucocytes. Orig. Mag. ×600.
Fig. 8(a, b) : No. 6311, 12 hr postchallenge. Degenerated surface epithelial cells, among a few 
of them containing the fluorescing bacilli. Orig. mag. ×600.
Fig. 9(a, b) : Lower large intestine in No. 6300, 12 hr postchallenge. Slightly inflammated area 
in the lamina propria situated below the surface epithelial cells moderately contain-
ing the fluorescing bacilli. Orig. mag. ×600.
Fig. 10(a, b) : Upper large intestine in the same case as Fig. 9. Surface mucosal lesion heavily 
containing the fluorescing bacilli in epithelial cells. No bacilli were detected in 
lamina propria. Orig. mag. ×400.
Fig. 11(a, b) : No. 5784, 3 days after inoculation. The epithelium markedly degenerated and fell 
off. The fluorescing bacilli were predomantly found in surface and also in deep 
epithelial cell of crypts (left lower) but a few in lamina propria (right lower). Orig. mag. ×600.