EPIDEMIC DIARRHEAL DISEASE OF SUCKLING MICE

IV. Cytoplasmic Inclusion Bodies in Intestinal Epithelium in Relation to the Disease*‡

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In a previous report (1), Pappenheimer and Enders described the occurrence of intranuclear inclusions in a large proportion of suckling mice with diarrhea (Fig. 1). The original stock in which the spontaneous disease had been prevalent for several years was virtually destroyed during the summer of 1946. Since then, new stock from several sources has developed spontaneous diarrhea, indistinguishable, as far as obvious signs are concerned, from the disease as it appeared in the original stock. The epidemiology of the disease as now prevalent is described in the preceding paper (2). In no instance have we succeeded in demonstrating intranuclear inclusions of the type previously described. However, many of the mice have had *cytoplasmic* inclusions in the epithelial cells of the small intestine.

In this study, evidence is presented bearing on the possible relation of these cytoplasmic inclusion bodies to the diarrheal disease as it has occurred in our colony subsequent to the introduction of new strains.

Methods

The entire small intestine was fixed in Zenker's fluid, without acetic acid, and embedded *in toto* so that the paraffin sections passed through the gut at various levels. As routine stain for the demonstration of the inclusions, Laidlaw's acid fuchsin-phosphomolybdic acid-orange G method has proven most satisfactory, although the bodies are also easily found in hematoxylin-eosin and in Giemsa-stained preparations.

Appearance of the Inclusion Bodies (Fig. 2).

The bodies are spherical, varying in size from 1 to 4 micra in diameter, sharply outlined, and sometimes, but not regularly, surrounded by a narrow clear halo. With the Laidlaw stain, they are intensely fuchsinophilic. In the larger forms, the center may appear rarefied. The position in the cell varies; the bodies may be found above, below, or to one side of the nucleus. There is usually only a single inclusion body to a cell, but occasionally, there may be

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several. They are present only in the cells over the summit of the villi and never in the cells at the base of the folds.

It is not uncommon, in the later days of the disease, to find free in the lumen of the gut, rounded, exfoliated cells in which the nucleus has become pycnotic and the chromatin massed in several fragments against the nuclear membrane. These cells frequently contain one or more fuchsinophilic bodies, identical in appearance with those found in the attached cells. Indeed, it is sometimes

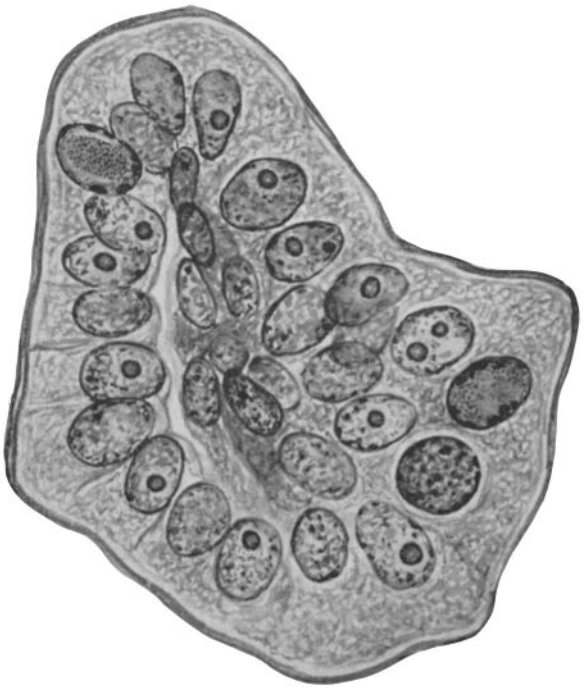

FIG. 1. Intranuclear inclusions in epithelial cells of small intestine. They are of two types, (*a*) spherical refractile, (*b*) diffuse granular. Laidlaw stain. × 1000.

possible to find degenerating, partially attached inclusion-bearing cells, or cells which have evidently just become dissociated, leaving a V-shaped depression over which the cuticular membrane is interrupted. It seems that the inclusion-containing cells tend to be cast off, leaving an intact epithelial layer behind.

In no case is there any inflammatory cellular reaction, nor are there any very definite changes in the epithelium as a whole. However, the presence of large numbers of inclusions does lead to slight disorganization in the alignment of the epithelial cells.

The inclusions have been found only in the small intestine, and in some cases seemed to be restricted to only one or several loops. Often long search is

required for their detection, so that failure to find them in some cases does not necessarily mean that they were not present.

With the Laidlaw stain, the granules of Paneth's cells stain intensely with acid fuchsin, and are very resistant to decolorization with alcoholic orange G. Their location at the base of the villi, and a slight difference in color, the Paneth granules having a slight purplish cast as compared with the vermilion red color of the cytoplasmic inclusions, enable one to distinguish them without difficulty.

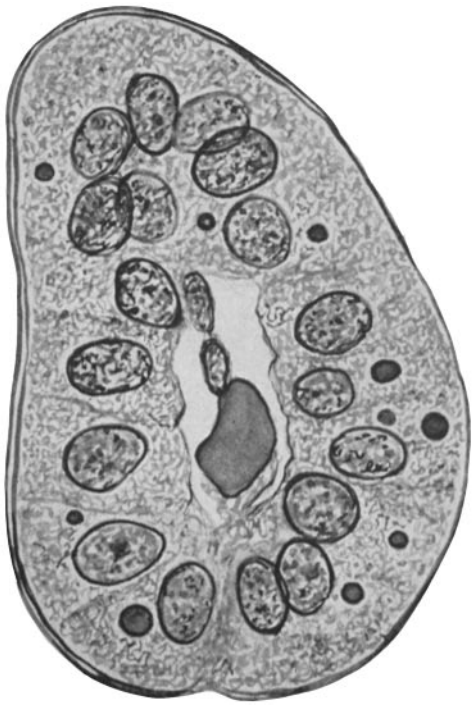

FIG. 2. Cytoplasmic inclusions in epithelial cells of small intestine. Laidlaw stain. × 1000.

More confusing is the frequent occurrence, especially in healthy mice a week or less old, of a somewhat different type of cytoplasmic inclusion. This was described and pictured in a previous report (1). It stains with Laidlaw, Giemsa, and eosin much as does the inclusion body described above, but with the Laidlaw stain, it holds the fuchsin much less tenaciously upon decolorization with orange G. The average size is much larger; the shape tends to be oval rather than spherical; it is surrounded by a wide clear space, and it is always located above the nucleus towards the intestinal lumen. When present, practically every cell of the villus contains such a body, and there is never more than one to a cell.

Although there is nothing to give a clue as to the significance of the type of body just described, it can usually be clearly differentiated from the cytoplasmic inclusions with which we are particularly concerned in this study. The dif-

ferential features of the two types will be obvious from the following tabular comparison:—

	Type A	*Type B*
Occurrence	Diarrheal mice (latent infection?)	Normal mice, rarely in diarrheal mice
Location in cell	Supra-, juxta-, or infranuclear	Supranuclear only
Size	Varies, 1 to 4 micra	Uniform 5 to 7 micra
Number	One or several in single cell	Only one to cell
Staining	Intensely fuchsinophile	Easily decolorized
Distribution	Found in individual cells, sometimes only after long search	Present in all surface cells of villus
Desquamation	Inclusions often present in desquamated cells	Not seen

Possible Relation of Type A Inclusions to Diarrheal Disease

In 57 mice of the Schwentker strain, with spontaneous diarrhea, which were killed between the 10th and 16th days, cytoplasmic inclusions were found in 31—an incidence of 54 per cent. Excluding 3 mice in which the sections were poor and only 2 or 3 jejunal loops examined without finding inclusions, the incidence would be increased to 57 per cent. In 32 normal mice of the same strain, no inclusions of Type A were found. In many of these cases, the examiner did not know whether or not the mouse had diarrhea until the completion of his study. In other strains (C, C3H, CFW, Harvard) the inclusions were less frequently found. Thus of 29 diarrheal mice of these four strains only 6 showed typical bodies. Since subsequent experience has taught that the inclusions are present in numbers only in the early days of the disease, and since these spontaneously diseased mice were sacrificed at various times after onset of symptoms, the low incidence of inclusions may be accounted for in this way.

Cytoplasmic Inclusions in Experimentally Infected Mice

Experiments describing in detail the production of diarrheal disease in suckling mice by oral administration of crude or filtered intestinal suspensions will be described elsewhere. Here we shall report only on the occurrence of inclusions in relation to the experimentally produced disease.

The first experiment involving 85 mice of the Schwentker strain, yielded equivocal results.

Experiment 1.—The mice were divided into three groups. Sixteen received crude extract of diarrheal intestine by mouth at the age of 4 days. All developed diarrhea, but many had recovered when killed for examination at the age of 17 to 21 days. In this group, cytoplasmic inclusions were found in only 2 mice, and inclusion-containing desquamated cells in one or two others. Twenty-seven mice received crude extract which had been boiled for 20 minutes. Of four litters, one developed diarrhea—probably an accidental infection. The other three litters remained free from obvious signs of the disease. Inclusions in this group, all killed at the age of 21 days, were found in 12 of the 27 (45 per cent).

In a third group, consisting of 42 mice, the crude extract was rendered bacteria-free by treating it with penicillin and streptomycin. The mice developed mild diarrhea; many had recovered when they were sacrificed at 21 days. Cytoplasmic inclusions were found in 8 of the 42 mice.

In summary, the incidence of inclusions in 64 mice with diarrhea was 11 or 17 per cent, whereas in the 21 mice which showed no signs of the disease, it was 47 per cent. There appeared to be no correlation whatever between the occurrence of diarrhea and the presence of cytoplasmic inclusions.

It should be said that with one or two exceptions, the inclusion bodies were very scarce, and often found only after long study of the sections. In a number of mice, they were seen only in castoff cells lying free in the lumen. It is probable that examination at an earlier stage before the affected cells had been exfoliated, would have yielded quite different results. Nevertheless, the presence of inclusions in a fairly large percentage of mice which had been fed with heated suspension and had failed to develop the disease, threw doubt upon their significance as a specific feature of the disease. There remained however, the theoretic possibility that the mice had acquired an inapparent infection while they were under observation. Since one of the four litters given heated extract did actually develop diarrhea in the course of the experiment, this possibility could not be excluded.

Experiment 2.—Four litters, in all comprising 26 mice, were fed crude suspension of diarrheal feces, at the age of 7 and 8 days, and were sacrificed 5 days later. At this time, two of the four litters showed the usual signs of diarrhea, two litters remained apparently well. Cytoplasmic inclusions were found in 22 of the 26 mice (85 per cent); they were present in 10 mice which did not have signs of diarrhea, although they had been given potentially infective material. This suggests that these may have had the disease in inapparent form.

As controls, 36 mice received a suspension of feces from normal stock which has been free of the disease, and has been maintained in a separate room under special precautions. None developed diarrhea, and in none were cytoplasmic inclusions found.

The experiment indicates that the feces of normal mice do not contain the factor responsible for the cytoplasmic inclusions.

Experiment 3.—Nine mice from three different litters were fed crude extract, and 9 mice were given boiled extract. One mouse from each litter was examined after 2, 3, and 6 days. Of those given crude extract, 5 developed diarrhea; those receiving boiled extract remained well.

In the former group, 6 of the 9 showed cytoplasmic inclusions; they were found also in 2 of the 9 mice which had received the boiled extract, though in small numbers and only after prolonged search.

Experiment 4.—To eliminate the possibility that some of the mice might have become infected while under observation, the experiment was repeated. The mice receiving the heated extract were kept isolated in a separate room which had been free from the disease. All the 20 mice given heated extract remained well, and in none were typical inclusions found. Seventeen mice received crude unboiled extract. Five were killed and examined before the onset

of symptoms, and showed no inclusions. The remaining 12 developed diarrhea, and in 11 of these, typical inclusions were demonstrated.

Time of Appearance of Inclusions in Relation to Diarrheal Disease (Fig. 3)

When the material from both spontaneous and experimentally produced disease is analyzed with respect to the relation between the presence of inclusions and the day of the disease, it is strikingly shown that inclusions are regularly found only in the early stages of the infection. The experimental infection is particularly informative in this respect. Whereas 32 of 33 mice

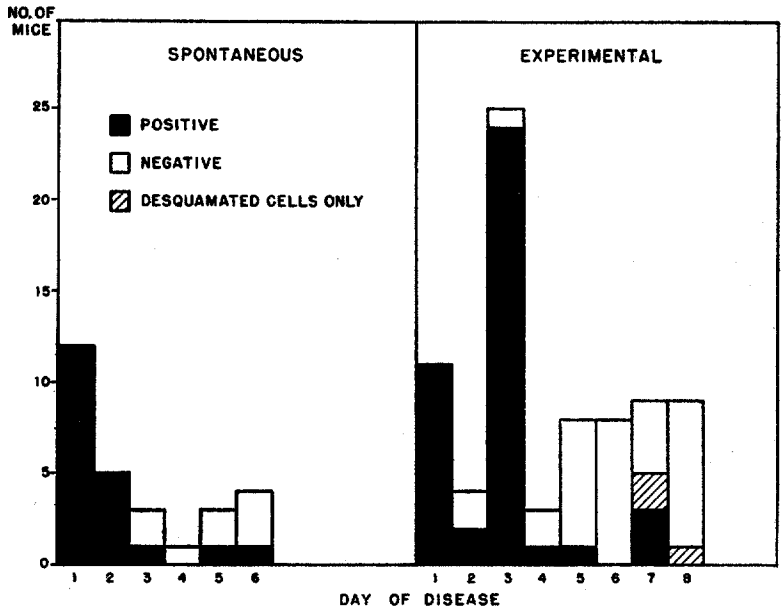

FIG. 3. Occurrence of cytoplasmic inclusion bodies by day of disease

(97 per cent) killed on the 1st, 2nd, or 3rd day of the diarrheal illness, showed inclusions, only 7 of 21 sacrificed on the 4th to the 8th day were recorded as positive; in 3 of these, the inclusions were seen only in desquamated cells lying free in the lumen. A similar difference was found in the spontaneous cases. Of 19 mice killed on the 1st and 2nd days after appearance of signs, 17 (89 per cent) had inclusions; these were present in but 3 of 11 sacrificed between the 3rd and 6th days, and only in sparse numbers.

It is thus important to search for the inclusions in the very early stages of the disease.

Gram-Positive Coccoid Bodies in the Intestinal Contents

Almost from the beginning of our studies, we have noted the presence, usually in enormous numbers, of Gram-positive coccoid bodies in the contents

of the small intestine. Although they may occur in stock mice which have shown no diarrheal symptoms, they are present with far greater frequency in the intestines of the diarrheal animals, and appear to be in some way correlated with the disease. This is brought out more clearly by our study of the experimental material. Thus, in Experiment 4, coccoid bodies were noted in 14 of 17 mice which had received crude extract and had developed diarrhea in consequence; and they were absent in 20 non-diarrheal controls given sterile heated extract. The 3 negative cases in the infected group were sacrificed on the 1st day of the disease.

The presence of these coccoid bodies can be recognized even under the low power of the microscope because, unlike the normal intestinal bacteria, they adhere in a fuzzy fringe to the epithelial surface. With the immersion lens, they are found to align themselves in a single row against the cuticular border of the cells. Unsuccessful attempts have been made to cultivate these organisms on the usual laboratory media. At this time, we wish merely to record their frequent and rather characteristic occurrence in this disease, without attempting further to assess their significance.

DISCUSSION

That there is a relation between the occurrence of cytoplasmic inclusions and the diarrheal disease of suckling mice now prevalent in the laboratory, can hardly be doubted. In the spontaneously occurring cases, inclusions were demonstrated in approximately 60 per cent; and they were invariably absent in stock of the same strain kept in a room which remained free from the infection.

In experimentally infected mice, the results are equally convincing. In 47 mice receiving crude unboiled extract of diarrheal intestine, 39 or 83 per cent were found to have typical cytoplasmic bodies. They were found in but 2 of 65 controls without diarrhea, including 36 given normal intestinal extract and 29 given boiled diarrheal extract. The 2 positive cases which occurred in the last group, we are inclined to attribute to some undetected error in labelling bottles or sections, or more probably, to the acquisition of an accidental infection during the period of observation.

It is true that the results in the first series of 87 mice were less clear cut, and that inclusions were found in a considerable number of mice that had been given boiled and presumably non-infective intestinal extract. We have no explanation for this discordant result, but the fact that one of the four litters developed diarrhea while under observation, suggests that they may have become accidentally infected in the course of the experiment. When precautions were taken to keep the animals in a non-infective environment throughout the experiment (Experiment 4), no inclusions were to be found.

The presence of inclusions in certain litters which had been given potentially infective material, but which failed to manifest signs of diarrheal disease, also

remains to be explained. Although it seems probable that these mice acquired an inapparent infection, no proof of this is at hand. It would have been interesting to feed intestinal extracts of these mice to normal mice, but this has not been done.

Another interesting problem in connection with these observations, to which no answer is forthcoming, is the relation of the current disease, characterized by cytoplasmic inclusions to the disease which prevailed in the laboratory stock prior to its accidental destruction during the summer of 1946 (3). The inclusions found before that critical event were exclusively intranuclear. Are we now dealing with another diarrhea virus, not to be distinguished by its obvious manifestations from that previously studied? No material for carrying out cross-protection experiments is available, but one is inclined to believe from the data thus far obtained that there are at least two viral agents capable of inciting diarrhea in suckling mice.

CONCLUSIONS

1. Cytoplasmic inclusions were found in the epithelial cells of the small intestine in a major proportion of suckling mice suffering from the spontaneous or experimentally produced diarrheal disease now prevalent in this laboratory.

2. They were not found in healthy stock mice of corresponding age.

3. Feeding of intestinal extract from healthy mice did not produce diarrhea or inclusions.

4. Feeding of boiled extract from diarrheal mice did not lead to the formation of cytoplasmic inclusions, when precautions were taken to prevent accidental infection.

5. The inclusions were regularly present only in the first few days of the disease. The inclusion-bearing cells desquamated. There was no inflammatory reaction.

6. Attention is called to the frequent presence of large numbers of Gram-positive coccoid bodies in the intestinal contents of suckling mice with diarrhea.

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