REPORT No. FE-382-4 (Annual Report)

GRANT No. DA-CRD-AFE-S92-544-69-G150

THE NATURE AND MODE OF ACTION OF LOCAL ANTIBODY IN INTESTINE

by

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October 1970

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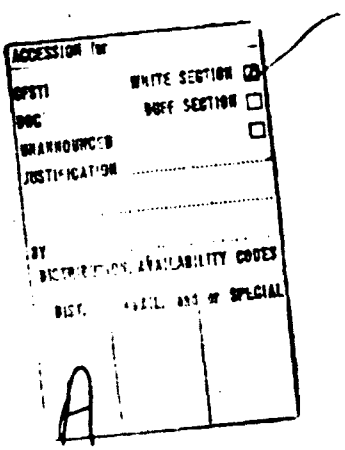

Unclassified
Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) The Kitasato Institute Tokyo, Japan	2a. REPORT SECURITY CLASSIFICATION Unclassified 2b. GROUP

3. REPORT TITLE

THE NATURE AND MODE OF ACTION OF LOCAL ANTIBODY IN INTESTINE (U)

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)
Annual Report, No. 4, 13 February 1969 - 12 February 1970

5. AUTHOR(S) (First name, middle initial, last name)

Akira Ghoda

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
26 January 1972	14	0

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
DA-CRD-AFE-S92-544-69-G150 b. PROJECT NO. 2N061102B71D c. Task 00 116FE d.	FE-382-4 9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)

10. DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	U.S. Army R&D Group (Far East) APO San Francisco 96343

13. ABSTRACT

Mouse clostrum IgA were prepared from the first day milk and 2-5 days pooled milk, and IgG and IgM were prepared from adult mice sera which were obtained from mice immunized with soluble cell-free cholera vaccine. Rabbit antisera against those immunoglobulins were prepared. Anti-IgA prepared from the first day milk was better than that of prepared from the 2-5 days pooled milk. The first day milk was rich in IgA and 2-5 days milk was rich in IgM. Almost all of feces of mice which were immunized with cell-free cholera vaccine contained IgA while very small number of feces from non-immunized mice contained IgA. IgM was not detected in feces but was detected in some of the extract of intestinal tissues from immunized mice. No IgG was detected in feces or in extracts of intestinal tissues regardless of whether the mice was immunized or not. (Author)

DD FORM 1473 (PAGE 1) (REPLACES DA FORM 1473, 1 JAN 66, WHICH IS OBSOLETE FOR ARMY USE.)

Unclassified
Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Intestinal infections						
Immunization						
Shigella						
Ligated loop						
Antibody						
Coproantibody						
Vaccine						
Cholera						
Toxins						
Animal						
Rabbits						
Mice						
Germ-free animals						
Japan						

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DA PROJECT/TASK/WORK UNIT No. 2N061102B71D 00 116FE

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ABSTRACT

Mouse clostrum IgA were prepared from the first day milk and 2-5 days pooled milk, and IgG and IgM were prepared from adult mice sera which were obtained from mice immunized with soluble cell-free cholera vaccine. Rabbit antisera against those immunoglobulins were prepared. Anti-IgA prepared from the first day milk was better than that of prepared from the 2-5 days pooled milk. The first day milk was rich in IgA and 2-5 days milk was rich in IgM. Almost all of feces of mice which were immunized with cell-free cholera vaccine contained IgA while very small number of feces from non-immunized mice contained IgA. IgM was not detected in feces but was detected in some of the extract of intestinal tissues from immunized mice. No IgG was detected in feces or in extracts of intestinal tissues regardless of whether the mice was immunized or not.

THE PURPOSE OF THE STUDY

Studies on infection and immunity in intestinal infections have been carried out by many researchers and yet little is known about these mechanisms. One of the reason was that the method of animal experiment was not well developed, therefore, the exploration of "the real status of the disease" by animal experiments was not possible. Fortunately, however, many methods and techniques have recently been developed for this purpose. Formal et al have studied the mechanism of the infection and the active immunization of Shigella using guinea pigs and monkeys: we have studied them by the modified ligated ileal loop method of adult rabbits: Burrows et al and Freter have studied the mechanism of induction of disease and the immunity of cholera by our method and by suckling rabbits.

From many experiments using those methods, hitherto unknown mechanisms and facts were revealed, but there still remain many important unsolved problems. For instance, it became clear that the circulating agglutinating antibody has no correlation with the protective activity in human and experimental animals in dysentery, and it plays a minor tole, if any, in cholera, and that normal intestinal flora play an important role at the beginning of the intestinal infection.

On the other hand, re-examination of the utilization of oral vaccines for the immunization of intestine has been highlightened by the lower incidence of side effects and dissatisfaction with the ineffectiveness of the conventional vaccination method for the protection of the intestinal infection after the success of Sabin's vaccine. Thus, clarification of the role and mode of action of local immunity in intestines became the important subject.

But, the nature of the local immunity including so-called cellular immunity is obscure and the only immune mechanism so far known is the coproantibody. Burrows et al and Freter studied coproantibody in human and experimental cholera, and we studied it in human and experimental dysentery. In those experiments, it was shown that the appearance of coproantibody was faster than that of the circulating antibody: that the oral administration of vaccine was much effective than any other way of administration of vaccine for the production of coproantibody: and that the titer of coproantibody paralelled with the protective capacity in immunized persons and in animals. Therefore, the possible role of coproantibody in local protection from intestinal infections has been suggested by the above mentioned researchers. However, Formal reported that he could not demonstrate the correlation in his studies on experimental shigellosis using monkeys.

Moreover, the chemical nature and the mode of action of coproantibody are still unknown and the correlation with other mechanisms such as interferon which is known to be produced in cells by viral infections have not been studied.

One of the drawbacks in the study of coproantibody was the difficulty in analysis of the response because of the comlicated nature of the response due to the existance of various intestinal normal flora. But this problem will be solved by the use of washed ligated ileal loop or germfree or other

gnotobiotic animals.

The purpose of the study is to explore the chemical nature of the specific action of coproantibody using simplified system, such as the washed ligated ileal loop or germ free animals, and Vibrio choelrae, its toxin, Vibrio parahaemolyticus and Shigella as inducing agents, and to establish the basis for the evaluation method and the development of oral vaccine.

MATERIALS AND METHODS

1. MATERIALS

a. Strains of Organisms. For cholera experiments Vibrio cholerae biotype eltor, serotype Inaba V 86 and serotype Ogawa #17 were used, and for Shigella experiments Shigella flexneri 2a #11 was used.

b. Cell Fractions. Vibrio cholerae biotype eltor were grown on nutrient agar plates for 16 hours at 37C, harvested in saline to the decity of 180 billion organisms per ml. The suspension was treated with urea and then desrupted by French press (Ohtake) for three times in 4C, and the supernatant and sediment was separated by centrifuge. The saturated ammonium sulfate solution was added to the supernatant and precipitates formed at 30% saturation were separated by centrifuge. The supernatant was further treated with ammonium sulfate solution and precipitates obtained at 52% saturation were separated. Those precipitates were dialyzed against distilled water. The fraction obtained at 30% saturation was called F30 hereafter and that of obtained at 52% saturation was called F52, and affix I and O before F30 or F52 indicates that those fraction was derived from Inaba and Ogawa respectively. The protein contents of those fraction were measured by Lawry's modification of Folin-Ciocalteu phenol reagent and nitrogen contents were measured by micro Kjerdahl's technic. Fractions were diluted to contain 5 µg dry weight per ml and then lypholized. Lypholized materials were restored to their original volume before inoculation.

c. Animals.

i. Conventional Mice. Five weeks to nine weeks old ICR/JCL strain of mice were used.

ii. Germfree Mice. Five weeks old ICR/JCL strain of germfree mice were used. Mice were kept in vinyl isolators and fed with sterile pellet. Drinking water was supplemented with vitamine complex, but when it was necessary to make mice vitamine deficient state vitamine complex was omitted from drinking water.

iii. Rabbits. Coccidium-free sibmating 8th generation Japanese white rabbits were used throughout experiments.

2. METHODS

a. Preparation of Coproantibody. Feces or intestines of animals

were taken out and to each 1 g of feces or tissues 1 ml of sterile saline was added. The material was homogenized with a glass homogenizer and the material was frozen in a freezer of a refrigerator. The material was taken out to room temperature next day and thawed gradually at room temperature, and then frozen again. This procedure was repeated 10 times and then centrifuged at 3,000 rpm for 30 minutes. The supernatant was used as the original crude coproantibody and agglutinin titer was measured by the conventional agglutination technic. The crude coproantibody was stored at frozen state.

b. Purification of Coproantibody. Crude coproantibodies obtained from different rabbits in the same experiment were pooled and measured for their agglutinin titers and then fractionated by gel filtration using Sephadex G100 or G200 packed in a 1.7 x 90 cm column and Tris-borate buffer, 0.2 M of pH 8.3, as the eluent.

c. Preparation of Mouse IgA. Eight (8) weeks old male and 7 weeks old female conventional mice were mated and 220 suckling mice were obtained at the first trial and 200 suckling mice were obtained at the second time. The first group of suckling mice were nursed for one day and the second group of mice were nursed for 5 days and then sacrificed and obtained clotted milk from their stomach. Clotted milk was suspended in phosphate buffer of pH 7.2, crushed, and then centrifuged at 15,000 rpm for 15 minutes. Clear zone of solution between sedimented casein and floating fat was drawn carefully and it was dialyzed against 0.1 M Tris-HCL-0.2 M NaCl buffer of pH 8.0 for one week. The material was then run through Sephadex G200 column with the same buffer described in the above. The first peak of eluent was dialyzed against phosphate buffer of pH 8.0 for 2 days. The material was then run through DEAE cellulose column with phosphate buffer and the peak obtained between 0.08 M and 0.1 M eluent was collected. The material was concentrated and dialyzed. This procedure was repeated 3 times. The material was used as the mouse IgA globulin. The material was kept in frozen state.

d. Preparation of Mouse IgG. ICR/JCL strain of 5 weeks old male mice were twice injected intraperitoneally with 0.1 ml of the solution which contained 1 mg dry weight of IF30 fraction at 6 days interval, and after 7 days of the second injection mice were sactificed by drawing blood from heart. Serum was separated and salted out 3 times with ammonium sulfate (50% saturation) and sediments were separated by centrifuge. The sediment was desolved in 0.01 M phosphate buffer of pH 8.0 and DEAE column chromatographic separation was carried out. The first peak of the eluent was separated and further purified by Sephadex G200 and the second peak was separated. The material was further purified by preparative electrophoresis using polyacrylamide as supporting medium. The purified IgG fraction was stored in frozen state.

e. Preparation of Mouse IgM. ICR/JCL strain of 5 weeks old male mice were once injected with 0.1 ml of solution which contained 1 mg dry weight of IF30 fraction intraperitoneally, and after 5 days of the injection mice were sacrificed by drawing blood from heart. Serum was separated and salted out 3 times with ammonium sulfate (50% saturation) and sediments were separated by centrifuge. The sediment was desolved in 0.01 M phosphate buffer of pH 8.0 and gel filtration using Sephadex G200 was carried out. The first peak of eluent was again run through Sephadex G200 and concentrated.

The purified IgM fraction was stored in frozen state.

f. Preparation of Anti-Mouse IgA Rabbit Serum. One half (0.5) ml of purified IgA fraction of mouse was injected into rabbit's food pad with Freund's complete adjuvant and after 14 days of the injection the second injection was made. After 30 days of the second injection rabbits were sacrificed by drawing blood. Serum was separated and was absorbed with serum from normal suckling mice. The purified anti-mouse IgA rabbit serum was stored in refrigerator.

g. Preparation of Anti-Mouse IgG Rabbit Serum. One half (0.5) ml of purified IgG fraction of mouse was injected into rabbits food pad with Freund's complete adjuvant and after 21 days of the injection the second injection was made. After 30 days of the second injection rabbits were sacrified by drawing blood from heart. Serum was separated and absorbed with normal suckling mice serum. The purified anti-mouse IgG serum was stored in refrigerator.

h. Preparation of Anti-Mouse IgM Rabbit Serum. One (1) ml of purified mouse IgM was injected into rabbit's food pad with Freund's complete adjuvant and after 19 days of the injection the second injection was made. After 30 days of the second injection rabbits were sacrificed by drawing blood from heart. Serum was separated and was absorbed with serum and clotted milk from suckling mice. The purified anti-mouse IgM rabbit's serum was stored in refrigerator.

i. Immunoelectrophoresis.

i. Preparative Immunoelectrophoresis. Preparative immunelectrophoresis was performed using polyacrylamide and veronal buffer pH 8.2 at an ionic strength of 0.025, under a current of 100V 30 mA for 50 minutes.

ii. For Identification Purpose. Immunoelectrophoresis for identification was carried out using acetate membrane and veronal buffer of pH 8.3 at an ionic strength of 0.025, under a current of 100V 30 mA for 45 minutes. The membrane was stained by Ponceau 3R stain.

j. Immunization of Mice. Mice were immunized twice intraperitoneally with a solution of fraction which contained 5 µg dry weight of fraction per 0.2 ml with 5 days intervals.

k. Oral Immunization of Rabbits. Rabbits were immunized orally with aceton dried organisms at 2 days intervals. The dose and times of vaccine given was varied by organisms and experiments.

EXPERIMENTAL RESULTS

1. Mouse IgA and Anti-Mouse IgA Rabbit Serum. At the first experiment, 6 ml of clotted milk was obtained from 220 suckling mice and from that 0.5 ml of purified globulin was obtained. The globulin contents in this material was very small and we did not know whether this material can induce antibody in rabbits or not, but we started immunization of rabbits with this material

anyway. And at the same time the collection of blood from suckling mice was started. After 30 days of the second injection of IgA, the rabbit was sacrificed by drawing blood. Serum was separated and then the same volume of serum obtained from suckling mice was added to the serum. The mixture was kept at 37C for overnight and then centrifuged at 6,000 rpm for 1 hour. The absorbed serum was used as the anti-mouse IgA rabbit serum.

This serum gave a strong precipitin line against first day milk of mouse but gave weak line against pooled milk of 1-5 days. This antiserum gave faint line against sera both to cholera vaccine immunized and non-immunized adult mice. The antiserum was compared with anti-mouse myeloma IgA rabbit serum which was kindly supplied by Dr. Matsuhashi of Tokyo University. Unfortunately his antiserum has very low titer and we could not draw conclusion, but there is some difference between these two anti-mouse IgA rabbit serum.

When these sera were tested against milk of immunized and non-immunized mice our serum gave only one line while anti-mouse myeloma IgA serum gave three precipitin lines and the most faint line diffused with that of against our anti-serum. Also when these sera were tested against sera of adult mice anti-mouse myeloma serum gave three precipitin lines while our serum gave a very faint diffuse precipitin line. When our serum was tested by immunoelectropnoresis against serum and first day milk of mouse there appears one distinct line comparable to IgA.

The second rabbit which was immunized with the second batch of IgA which was obtained from 1-5 days milk by the same method and schedule as described in the above was sacrificed and obtained the serum. This anti-mouse IgA rabbit serum was also tested against 1st day milk, 2-5 days milk and mouse serum. 1st day milk gave a very faint precipitin line while 2-5 days milk and serum gave three precipitin lines.

The first and the second antiserum was tested against fecal extracts which were obtained from IF-30 immunized adult mice. The first serum gave a strong precipitin line with most of the extracts while the second serum gave very faint line to some of the extracts. Therefore, it is conceivable that the first antiserum is the true anti-mouse IgA antiserum and the second serum contains not only anti-IgA which is weak but also contains other factors. The study of the nature of the other factors is in progress.

2. <u>IgG and anti-IgG rabbit serum.</u> Mouse IgG and its anti-rabbit antiserum was prepared by the method and procedures described in the section of Materials and Methods.

The serum gave a strong distinctive precipitin line against sera of cholera vaccine immunized and non-immunized adult mice but no precipitin line was formed against fecal extracts of the same animals. The extracts of small and large intestine of mice were tested against the serum. Some of the extract gave precipitin line but most of the extract did not form any precipitin lines.

The first day milk and 2-5 days milk of mice were tested against the serum. Both of the milk formed strong precipitin lines and that precipitin lines

fused with that of formed against the second batch of anti-mouse IgA rabbit serum.

3. IgM and Anti-Mouse IgM Rabbit Serum. Mouse IgM and its anti-rabbit antiserum was prepared by tha method and procedures described in the Section of Material and Methods.

The serum was tested against sera of cholera vaccine immunized and non-immunized adult mice. A strong distinctive precipitin line was formed. The first day milk, 2-5 days milk, extracts of small and large intestines were tested against the serum. Both of the milk gave a strong precipitin line against the serum and some of the extracts of the small and large intestines gave a strong precipitin line but most of the extracts gave no precipitin line.

Those result indicate that the first batch of anti-mouse IgA rabbit serum containes pure anti-mouse IgA while the second batch still containes anti-mouse IgA and IgM, and the first day milk contsines more IgA than the second pool of milk and that will be the reason why the first batch of anti-mouse IgA was better than that of the second batch.

It is interesting to note that IgG and IgM was proven in some of the extracts of intestines. The condition and the time of the appearance of those immunoglobulin in the intestines will be studied in the near future.