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Vibriocidal Activity, Immune Globulin Producing Cells and Immune Globulin Levels in *Theropithecus gelada* After Administration of a *Vibrio cholerae* Antigen

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Summary. Geladas were fed or injected with an antigen that contained Burrows' type 2 cholera toxin. Rising agglutinin and vibriocidal titres were observed in the serum, peripheral and mesenteric lymph nodes, spleen and lymphatic tissue of the upper intestine. Oral administration stimulated a more intensive vibriocidal activity in the mesenteric lymphatic nodes and intestinal lymphatic tissue, and within a shorter time than parenteral injection of the same antigen. Immune globulin synthesis paralleled largely the number of immunologically active cells. The agglutinin titres reflected the level of immune globulins and the numbers of globulin producing cells, whereas vibriocidal titres appeared independent of both. In terms of antibody site serum IgG was weight for weight more vibriocidal than serum IgM.

INTRODUCTION

The relationship of cholera antigens and antibodies to immune globulins (Igs) and immune globulin producing cells (IPCs) of the lymphocyte-plasma cell series has been studied in primates by Felsenfeld and collaborators (Felsenfeld, Felsenfeld, Greer and Hill, 1966a; Felsenfeld, Hill and Greer, 1966b; Felsenfeld, 1966; Greer and Felsenfeld, 1966; Felsenfeld, Greer and Felsenfeld, 1967). Three antigens were employed in these experiments: (1) a phenolized cholera vaccine containing 8×10^9 or more vibrios per ml; (2) a lipopolysaccharide prepared according to Watanabe and Verwey (1965); and (3) a supernate separated from cholera vibrios after disruption of the bodies of the organisms by the aid of ultrasonic vibration. The last corresponded to cholera toxin type 2 of Burrows (1965) and Burrows, Musteikis, Oza and Dutta (1966). The antigens were administered parenterally or through a duodenal tube.

Vibrio cholerae agglutinating and toxin neutralizing antibodies were examined in the blood serum, intestinal contents, lymph glands, and spleen of each animal. Immunoglobulin M (IgM) and immunoglobulin A (IgA) appeared early after cholera vaccination. Agglutinins were present in all three classes of Igs tested, IgM, IgA and IgG. IPCs were most active in collections of lymphoid cells nearest the site of the inoculation; for example, they were more numerous in peripheral lymph nodes when the antigens were given parenterally and in the gut after oral application. Serum IgG seemed to play a major role in neutralization of toxin, but in the intestines other Igs, especially IgA, played a principal part. Vibriocidal antibodies, however, were studied only in the blood serum. Burrows has pointed out repeatedly (Burrows, 1965; Burrows *et al.*, 1966; Kasai and Burrows, 1966) that cholera immunity is both anti-toxic and anti-bacterial. It seemed,

therefore, that a study of the vibriocidal activity of the immune globulins in the intestines and in the lymphatic tissue was indicated.

Vervets (African green monkeys, *Cercopithecus aethiops*) and patas (*Erythrocebus patas*) had been used in the previous experiments. These are relatively small animals. Since separation of sufficient amounts of Igs from the lymphatic glands and intestinal lymph follicles of these small animals presented considerable difficulties, gelada baboons (*Theropithecus gelada*) were selected for further studies.

MATERIALS AND METHODS

Vibrio supernate

This was prepared according to Burrows (1965), by disruption of a *Vibrio cholerae* biotype El Tor strain (No. 17) suspension, containing $3–7 \times 10^{11}$ cells/ml in an ultrasonicator (Branson Apparatus Co., Stamford, Connecticut) at 2–4°. The few remaining intact organisms and the cellular debris were removed by spinning at 74,000 *g* in a Beckman ultracentrifuge model L at 2°. The supernate was kept at −20° for 2–3 days before administration.

Animals

Geladas of both sexes, 3–4 years old, weighing 6·5–14 kg, were used. These primates were free from infection as shown by bacteriological and parasitological examinations of the intestinal and respiratory tracts. The initial haemograms, cephalin flocculation, icterus index, alkaline phosphatase and SGP transaminase values were within normal limits for the species. Clinical examinations did not reveal pathological changes. There was no history of any disease for at least 6 months before the experiments.

The geladas were individually caged and divided into two groups of three. All animals were bled at the beginning of the experiment, then each individual in one group was given 2 ml cholera vibrio supernate of Burrows (1965) by duodenal tube ('oral administration'). Members of the other group of geladas were injected intramuscularly in the gluteal region with 0·5 ml of the same material ('parenteral inoculation').

On the 3rd *and* the 14th day after immunization, inguinal lymph nodes, upper mesenteric lymph nodes, part of the spleen, and a 1-cm wide elliptical strip of the proximal jejunum were excised under general anaesthesia with Sernylan (Parke, Davis & Co.). The bleeding from the spleen was stopped by application of oxidized cellulose. The jejunum was closed by applying inverting sutures with a continuous overlay. All animals survived and had recovered in 7 days.

Immune globulin separation and assay

Weighed amounts of tissues (about 0·2–2 g) were disrupted by ultrasonification in 0·01 M Na phosphate buffer, pH 7·8 at 4° and centrifuged at 30,000 *g* at 2°. The globulins from the supernates of these, or from serum samples were then separated on DEAE-cellulose columns by the stepwise gradient elution method of Oh and Sanders (1966), using 0·0–0·2 M solutions of NaCl in 0·01 N Na phosphate buffer at pH 7·8–6·5. The fractions were concentrated by exposure to a stream of cold air from a fan, then by immersion of the Viscose bags containing the fractions into 60 per cent polyvinylpyrolidone. The protein content of the fractions was determined with the biuret reaction. The fractions were run against rabbit anti-monkey IgG, IgA, IgM and whole serum in agar gel diffusion tests using the serial dilution method of Sharpless and LoGrippo (1965). Human globulins

and goat anti-human globulins (Hyland Laboratories) served as controls. All tests were run in triplicate.

Immune globulin producing cell count (IPC)

The tissues were prepared according to Dresser and Wortis (1965). Counts for IPC were made by the plaque technique of Jerne modified by Halliday and Webb (1965) and for IgG-producing cells by the method of Dresser and Wortis (1965). The calculations were made according to Wizzell (1966). A least three counts were made from each sample.

Agglutination tests

Agglutination tests were performed with homologous live organisms. The antigen–antibody mixtures were incubated at 50° for 2 hours, and read after an overnight's sojourn at 2–4°. Only 4+ and 3+ reactions were read as positive.

Vibriocidal activity (VA)

Because of the scarcity of material, the vibriocidal activity assays were carried out with Igs isolated from pooled tissue samples collected on the same day from all three animals belonging to one experimental group. The assay method was that of Finkelstein (1962) using two-fold dilutions of the respective Ig, $1–3 \times 10^3$ log-phase *V. cholerae* biotype El Tor strain No. 17, and 1:20 guinea-pig serum as complement. After 3 hours incubation at 37° in a water bath, 0·1 ml of each suspension was plated to yeast extract agar, pH 7·8, and the highest dilution inhibiting vibrio growth was read after incubation at 37° overnight. The titrations were carried out in triplicate.

RESULTS

Table 1 summarizes the results of the determination of Igs, the agglutination titres, and the vibriocidal activity in three Igs in the blood serum. Elevated IgA and IgM but not IgG levels in the serum were found 3 and 14 days after oral and parenteral adminis-

TABLE 1

RELATIONSHIP OF AGGLUTINATING AND VIBRIOCIDAL ANTIBODIES TO SERUM IMMUNE GLOBULINS IN GELADAS INOCULATED WITH CHOLERA VIBRIO SUPERNATE

Time of collection	Test	Oral administration			Intramuscular inoculation		
		IgG	IgA	IgM	IgG	IgA	IgM
Before	Ig (mg/100 ml)	810±23	115±11	239±14	827±29	105± 7	282±20
	Agg*	<20	<20	<20	<20	<20	<20
	VA†	<50	<50	<50	<50	<50	<50
3 days after	Ig (mg/100 ml)	725±14	188±10	327±18	815±38	217±10	415±21
	Agg	<20	<20	<20	<20	<20	<20
	VA	690±20	400±20	130±10	640±10	320±20	170±10
14 days after	Ig (mg/100 ml)	782±48	172±12	315±19	785±32	201±11	408±24
	Agg	80±10	80±10	90±10	320±30	170±40	320±20
	VA	640±20	320±20	170±10	800±40	690±30	400±20
18 days after	Ig (mg/100 ml)	812±52	122±11	252±25	761±25	185±11	301±21
	Agg	260±40	160±10	130±10	640±20	640±30	690±20
	VA	400±20	260±10	130±10	1280±60	800±40	640±20

* Reciprocal agglutination titre of 5 mg Ig adjusted to nearest 10±standard deviation.
† Reciprocal vibriocidal titre adjusted to nearest 10±standard deviation.

tration of the antigen. After 18 days, IgM returned to the levels observed at the beginning of the experiment. On that day IgA still remained somewhat elevated in the animals to which the antigen was given parenterally.

The agglutination titres of the three Ig classes were nearly identical and remained elevated at least to the last day (18th) of observation. The vibriocidal activity of IgG was greater than that of IgA which, in turn, exceeded that of IgM.

Table 2 shows the number of cells producing Igs and the concentration of Igs in the tissues. When the antigen was given orally, the number of IPCs was highest in the intestinal lymph nodes and relatively low in the peripheral lymph nodes. The opposite was observed after parenteral injection. Between the 3rd and 14th days after oral administration of the antigen, the number of IPCs and the concentration of Igs decreased in all

TABLE 2

RELATIONSHIP OF NUMBER OF IMMUNE GLOBULIN PRODUCING CELLS TO AMOUNT OF IMMUNE GLOBULINS IN TISSUES OF GELADAS INOCULATED WITH CHOLERA VIBRIO SUPERNATE

Tissue	Test	Days after	Oral administration			Intramuscular inoculation		
			IgG	IgA	IgM	IgG	IgA	IgM
Peripheral lymph node	IPC*	3	201±21	94±10	106±9	301±21	227±15	219±12
	Ig†		6·2±0·2	4·4±0·2	4·7±0·3	9·2±0·7	7·8±0·5	8·3±0·4
	IPC	14	101±10	65±7	52±4	337±21	281±20	115±12
	Ig		3·7±0·3	2·7±0·3	2·7±0·2	10·7±0·4	9·5±0·6	4·1±0·2
Spleen	IPC	3	306±32	207±18	128±11	218±15	175±9	131±8
	Ig		9·8±0·7	3·6±0·3	5·3±0·4	6·4±0·4	5·8±0·6	5·1±0·5
	IPC	14	203±15	124±7	101±8	349±25	237±21	298±20
	Ig		7·2±0·6	4·3±0·3	4·3±0·2	10±0·4	8·7±0·5	11·8±0·6
Mesenteric lymph node	IPC	3	310±20	215±15	121±13	188±20	121±10	104±10
	Ig		6·7±0·3	7·3±0·3	5·1±0·2	7·2±0·4	4·1±0·2	4·4±0·2
	IPC	14	173±10	127±12	125±13	308±22	295±25	265±21
	Ig		5·4±0·3	4·5±0·2	4·8±0·2	10·7±0·6	10·8±0·5	9·9±0·4
Intestinal lymph tissue	IPC	3	358±28	403±29	273±21	102±10	88±7	79±5
	Ig		13·1±1·1	14·1±1·1	10·8±0·9	3·8±0·3	3·5±0·2	3·3±0·2
	IPC	14	243±19	135±10	155±11	256±20	248±19	201±14
	Ig		8·1±0·3	4·5±0·2	6·7±0·3	7·9±0·2	8·8±0·4	8·1±0·4

* Immune globulin producing cells per 10^{-2} mg tissue ± standard deviation.
† μg immune globulin per mg tissue ± standard deviation.

tissues examined but the number of IPCs and the concentration of the Igs increased significantly in the spleen, mesenteric lymph nodes and intestinal lymphatic tissue during the same interval when the antigen was injected parenterally. A decrease of IgM in the peripheral lymph glands after 14 days was also noted following parenteral antigen administration. The proportion of the IPCs and Igs varied considerably within experimental error but probably not significantly.

Table 3 represents the results of IPC counts, Ig determinations, and vibriocidal titres in tissue samples collected on the 3rd and 14th days after immunization. Measurable vibriocidal titres appeared as early as the 3rd day after the administration of the antigen. The vibriocidal activity of the Igs was higher on the 14th day of the experiment than on the 3rd day after i.m. inoculation, whereas it either remained stationary within experimental error or decreased after oral administration. In the intestinal lymphoid tissue a particularly sharp increase of vibriocidal activity occurred between the 3rd and 14th day when the antigen was injected intramuscularly, but the vibriocidal activity had already reached

a high titre (1:2560) as early as the 3rd day after oral administration of the same vibrio supernate. There was no linear relationship between IPC count, the concentration of tissue Igs, and the vibriocidal titre of the tissue.

TABLE 3

RELATIONSHIP OF NUMBER OF IMMUNE GLOBULIN PRODUCING CELLS TO TOTAL IMMUNE GLOBULIN AND VIBRIOCIDAL ACTIVITY IN TISSUES OF GELADAS AFTER INOCULATION OF CHOLERA VIBRIO SUPERNATE

Specimen	Test	Days after	Oral administration	Intramuscular inoculation
Peripheral lymph node	IPC*	3	401 ± 26	748 ± 25
	Ig†		13·3 ± 1·2	25·3 ± 1·8
	VA‡		350 ± 20	400 ± 20
	IPC	14	218 ± 15	733 ± 28
	Ig		9·1 ± 0·6	24·3 ± 1·7
	VA		320 ± 30	690 ± 40
Spleen	IPC	3	641 ± 31	524 ± 28
	Ig		18·7 ± 1·6	17·3 ± 1·5
	VA		640 ± 30	320 ± 18
	IPC	14	426 ± 22	884 ± 41
	Ig		15·8 ± 1·1	30·5 ± 2·1
	VA		400 ± 20	640 ± 30
Mesenteric lymph node	IPC	3	646 ± 30	413 ± 26
	Ig		19·1 ± 2	15·7 ± 1·2
	VA		690 ± 30	260 ± 20
	IPC	14	425 ± 20	868 ± 40
	Ig		14·7 ± 1·2	31·4 ± 2·2
	VA		400 ± 30	870 ± 40
Intestinal lymph tissue	IPC	3	1034 ± 75	269 ± 19
	Ig		38 ± 2·3	10·6 ± 0·8
	VA		2560 ± 100	170 ± 10
	IPC	14	523 ± 26	705 ± 29
	Ig		19·3 ± 1·3	24·8 ± 1·4
	VA		1280 ± 60	1780 ± 80

* Immune globulin producing cells per 10^{-2} mg tissue ± standard deviation.
† μg immune globulin per mg tissue ± standard deviation.
‡ Reciprocal vibriocidal titre of activity per 5 mg Ig adjusted to nearest 10 ± standard deviation.

DISCUSSION

The results of these experiments complement observations made in vervets and patas monkeys immunized with phenolized cholera vaccine (Greer and Felsenfeld, 1966), toxin (Felsenfeld *et al.*, 1967) and vibrio lipopolysaccharide (Felsenfeld, 1966; Felsenfeld *et al.*, 1966a, b, 1967). In these experiments, oral introduction of the antigen elicited immediate and intensive agglutinating and antitoxic antibody formation in the gut, whereas the antibody titres in the intestines developed at a slower pace after intramuscular injection. In the present studies similar changes in vibriocidal activity were seen. The 5-mg amounts of IgG, IgA and IgM isolated from sera collected on the same day as the experiment from animals receiving the antigen by the same route appeared to have similar agglutinating titres, whereas the vibriocidal titre of the serum IgG had a tendency to be higher than that of IgA and IgM. Apparently serum IgM, at least under present experimental conditions, was less vibriocidal weight for weight than serum IgG. Technical limitations did not permit the study of vibriocidal activity of the individual Igs in the tissues. The vibriocidal activity of the combined Igs, however, did not show the linear relationship found with agglutinins. Neither was such a relationship apparent between

the IPC count and the vibriocidal titres. The amount of Ig formed seemed to depend on the number of IPCs. We are aware, however, of the limitations of the techniques at present available for the immunochemical investigation of small amounts of tissue.

In a current study of antibody formation to cholera toxin with the aid of fluorescent techniques (to be published), the distribution of the IPCs in the lymph nodes and spleen has been found to vary with the amount of the antigen and the interval between its application and the collection of tissue specimens. In spite of these difficulties in precisely determining the number of IPCs from small random samples of tissue, it is felt that the data presented in this paper permit the conclusion that the enumeration of IPCs in only one sample of tissue does not provide a dependable estimate of vibriocidin production by that tissue. It would be sheer speculation to attempt to explain the discrepancies observed during these studies in terms of lysozymes, 'humoral' factors, 'non-specific' bactericidins and other vibrio-destroying antibodies that might pass through chromatographic columns with Igs and not be detected by the methods routinely used to determine the purity of the Igs. It is hoped that further research will throw more light on this problem.

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