IMMUNITY IN VOLUNTEERS RECOVERED FROM NON-BACTERIAL GASTROENTERITIS [1]

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The afebrile type (1) of acute infectious nonbacterial gastroenteritis seems to be an extremely common diarrhea in the Northeastern United States. It occurs yearly in some families and communities (2, 3). Whether the recurrences are due to the early waning of immunity or to the existence of immunologically distinct agents has not been determined. Following an attack of gastroenteritis evoked by the Marcy strain (4), which is presumably a virus, an effective immunity develops in volunteers (1, 4–6), and in an earlier report the opinion was expressed that immunity probably does not persist for long (5). The present study shows that individuals who recovered from experimental Marcy gastroenteritis more than a year before were fully resistant to reinfection. It may well be, therefore, that the second explanation of the recurrences is the correct one and more than one antigenically distinct agent exists.

METHODS

Afebrile nonbacterial gastroenteritis can be highly contagious; numerous large epidemics have been reported (4, 5, 7, 8). Like the experiments previously done with the Marcy strain, therefore, this study was carried out in a closed state correctional institution, where volunteer subjects were individually isolated in well-equipped cells for periods of several weeks. Stringent precautions against accidental spread of the disease were enforced under nursing supervision.

Volunteers were healthy male inmates 21 to 30 years old who had no known exposure to gastroenteritis prior to their entry into the study. Their psychological stability was assured; almost all had undergone previous inoculation with control specimens such as autoclaved fecal suspensions or broth (6) or had participated in other oral inoculation experiments unrelated to nonbacterial gastroenteritis (9). The symptoms and signs they exhibited were therefore unlikely to be due to suggestion. Their feces contained no known enteric pathogens.

The inoculum was a supernate of watery feces collected from Volunteer 61 (T. H.), one of a group representing the 6th serial passage of the Marcy strain in volunteers. At 12:30 p.m. on July 2, 1950, he had abrupt onset of nausea and vomiting, followed by malaise, vertigo, and 27 episodes of watery diarrhea within the next 36 hours. Fecal specimens from eight bouts of diarrhea occurring between 7 a.m. and 6 p.m. on July 3, 1950, were collected in sterile glass screw-capped jars and frozen for storage at $-70°$C. within 10 minutes after the patient produced them. On April 12, 1951, they were thawed at $\backsim 37°$C. and centrifuged horizontally at 3000 r.p.m. at $\backsim 4°$C. for 45 minutes. The sediment was discarded and the supernate recentrifuged similarly for 30 minutes. The second supernate was thoroughly mixed, dispensed in 10-ml. amounts to 40 jars, and refrozen in the CO_2 box. Microscopic and cultural examination revealed no bacteria, and no infectious agent was detected by inoculation of tissue cultures and experimental animals, including monkeys and suckling mice.

The content of an appropriate number of jars was thawed from time to time for the experiments reported in the text. Some previous results with this inoculum have been published (1, 6). The inoculum for the present study was composed of different specimens than the pool made up of stools collected at other times from the same patient, employed for another investigation (10).

As in preceding studies (1, 4), inoculum was fed mixed with milk, and fecal specimens from each ill volunteer were inspected. Some specimens were collected for subsequent bacteriologic examination and inoculation of animals or tissue cultures.

Criteria for recognition of experimental illness were more strict than those employed in an early experiment (5). Experience has shown that the most common manifestations of the induced disease are watery diarrhea, abdominal cramps and pain, anorexia, nausea, and vomiting (1, 4, 6). In this study the diagnosis of Marcy gastroenteritis was based on the presence of watery diarrhea plus at least two of the other symptoms or signs. Anorexia could be objectively noted by physicians,

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TABLE I

Results of initial inoculations with stored aliquots of the inoculum from Volunteer 61

Inoc. No.*	Date inoc.	Duration of storage since collection on July 3, 1950		Dose ml.	No. of volunteers	Cases of gastro-enteritis
		Years	*Days*			
1(1)	12.17.51	1	168	7	7	6
3(1)	1.15.52	1	197	7	8	7
4(1)	6.23.52	1	357	7	1	1
5	3.28.53	2	270	1	8	3
7	4.17.53	2	290	7	6	6
8	5.18.53	2	321	3.5†	6	4
9	5.29.53	2	333	3.5‡	6	4
Totals: Volunteers fed 1 ml.					8	3
Volunteers fed 3.5 ml.					12	8
Volunteers fed 7 ml.					22	20
All volunteers					42	31

* Refers to occasions on which aliquots were selected at random, mixed, pooled, and used. (Inoculation 2 was an immunity test done approximately two weeks after onset of gastroenteritis and is omitted.)
† Mixed with 3.5 ml. of acute-phase serum pool. See text.
‡ Mixed with 3.5 ml. of convalescent-phase serum pool. See text.

nurses, or attendants since the volunteers were tray-fed; it could therefore be judged either as a sign or a symptom. The lack of symptoms and signs exhibited by these and other volunteers given noninfectious or sham inoculations under the conditions of the experiments has already been recorded (1, 6).

RESULTS

Various experiments in which volunteers from New York State correctional institutions were fed aliquots of the pooled inoculum from Volunteer 61 (see Methods) had shown that in spite of long storage in the dry ice box the inoculum continued to be highly infectious for previously uninoculated men (Table I, Inoculations 1, 3, 4, 5). Four subjects who had contracted the disease approximately 15 months before and one who had suffered an induced illness 9 months previously, revolunteered to be reinoculated to test whether they were immune (Table II). All five had exhibited the diarrheal syndrome of afebrile nonbacterial gastroenteritis with characteristic incubation periods after ingestion of initial doses of 7 ml. of the bacteria-free fecal supernate from Volunteer 61, portions of which were still on hand. Their illnesses have been mentioned in a published report and the least severe of them, Case E. H. (Volunteer 89), is among those represented graphically (1). The five men had remained well in the interim between their first exposure and their recruitment for reinoculation.

The design of the immunity trial was dictated by opportunity. The number of volunteers avail-

TABLE II

Reinoculation of five recovered volunteers with aliquots of the inoculum from Volunteer 61 to test active immunity

Initial inoculation (7 ml.) and illness				Reinoculation (7 ml.) to test active immunity				
						Interval since onset		
Volunteer	Inoc. No.*	Date inoc.	Onset of gastro-enteritis	Inoc. No.	Date inoc.	*Years*	*Days*	Result
86 (H. W.)†	1	12.17.51	12.20.51	6	4.10.53	1	112	No illness‡
73 (A. B.)	3	1.15.52	1.17.52	6	4.10.53	1	84	No illness
74 (A. E.)	3	1.15.52	1.16.52	6	4.10.53	1	85	No illness
78 (G. S.)	3	1.15.52	1.17.52	6	4.10.53	1	84	No illness
89 (E. H.)	4	6.23.52	6.27.52	6	4.10.53	0	287	No illness

* See Table I for explanation.
† This volunteer experienced a reinoculation with a 7-ml. dose of an aliquot of the same inoculum, on 1.2.52, and remained well (1).
‡ No symptoms or signs.

TABLE III

Tests of significance of differences between results of immunity trial and those of initial inoculations

	I	II	III	IV	V
Inoculation No.*	6†	7	8 and 9	7, 8, and 9	1, 3, and 4
Type	Reinoculation	Initial inoc.	Initial inoc.	Initial inoc.	Initial inoc.†
Dates	4.10.53	4.17.53	5.18.53 5.29.53	4.17.53 5.18.53 5.29.53	See Table I
Dose	7 ml.	7 ml.	3.5 ml.	7 ml. or 3.5 ml.	7 ml.
Not ill	5	0	4	4	2
Ill	0	6	8	14	14
P		0.0022	0.0204	0.0037	0.0010

* See Table I for explanation.

† The five volunteers reinoculated on the occasion of Inoculation No. 6 were among the 14 subjects who became ill following initial inoculations 1, 3, or 4 (Column V).

able to serve as controls was uncertain. We proceeded with the reinoculation on the premise that if a sufficient number of reinoculated men became ill, demonstration of the infectivity of the inoculum would be unnecessary, while if they did not become ill, the inoculum could subsequently be tested for infectivity in additional volunteers exposed for the first time. This was thought permissible because the inoculum to be used throughout was pooled and mixed before it was stored in 10-ml. aliquots, and the vials employed for each inoculation were selected at random from the dry ice box when needed.

The reinoculation consisted of feeding 7 ml. of pooled aliquots of the inoculum to each of the five men. This was the same dose of the same inoculum they had received before. None showed any symptoms or signs of illness (Table II).

During the next 2 months remaining aliquots of the inoculum were tested for potency in 3 new groups of subjects, none of whom had had prior exposure to Marcy gastroenteritis inoculum or known contact with the natural disease (Table I, Inoculations 7–9). Inoculation 7 was done in the usual way, but for Inoculations 8 and 9, serum was mixed with the fecal supernate. The reasons for this will be discussed later.

Each of the six volunteers fed a 7-ml. dose of fecal inoculum alone developed typical gastroenteritis (Table I, Inoculation 7). Of six men fed 3.5 ml. mixed with an equal volume of acute-phase serum from Marcy gastroenteritis volunteers, four became ill (Inoculation 8). The same outcome followed ingestion of 3.5 ml. of inoculum mixed with serum from volunteers convalescent from Marcy gastroenteritis (Inoculation 9). Each of the 14 cases that occurred among these 18 men fully met the criteria for diagnosis outlined above. A few were severe, most of them were of moderate severity, and a few were mild. An example of the latter is the case of W. B. (Volunteer 127), who swallowed 7 ml. of inoculum, developed mild abdominal cramps and borborygmi with watery diarrhea after an incubation period estimated at 64 hours, experienced anorexia and headache on the following day, during which the feces continued to resemble pea soup, then recovered.

The challenge of the five recovered volunteers was done 728 days and the three trials of infectivity were carried out 735, 766, and 777 days, after the fecal supernate had been pooled, mixed, and split into 10-ml. portions. The dose for Inoculations 8 and 9 was half that employed in the challenge of the recovered volunteers. Under these circumstances the attack rates obtained in the potency tests might tend to be lower than those expected if the infectivity of the inoculum had been determined in control subjects given the same dose at the same time as that fed the five men in the immunity trial. In spite of this possible bias, tests of significance show that the differences between the results of the immunity and potency trials respectively are highly unlikely to have occurred by chance variation alone (Table III).

If a 2 by 2 table is formed with the outcome of the immunity trial (Table III, Column I) and that of the potency test of an equal dose of 7 ml. (Column II) the value of P as used in the "exact treatment" (11) is 0.0022.[4] If the pooled results of initial inoculations with the 3.5-ml. dose (Column

[4] The value of P is obtained directly from Thompson's tables of the 4-variable psi-function (12), available in the Unpublished Mathematical Tables File of *Mathematical Tables and Other Aids to Computation.*

III) are similarly compared with those of the immunity trial, the difference is still significant (*i.e.*, $P < 0.025$). Analysis of the total number of induced illnesses among the 18 men in whom the infectivity of the inoculum was tested subsequent to the immunity trial in the same or lower dose (Column IV) gives a value of P of 0.0037. As a matter of interest P is also given for results obtained with a 7-ml. dose before the immunity trial was attempted (Column V).

These data are sufficient to show that the failure of the five men to suffer a second bout of Marcy gastroenteritis when they were challenged approximately a year after recovery from an initial attack was not due to lack of infectivity of the inoculum. Since experimental Marcy gastroenteritis is known to confer active immunity (1, 4–6), it can be deduced that immunity lasted during the interval between inoculations. No assumptions regarding the stability of the Marcy agent during prolonged storage need be made to support this conclusion. The findings are in accord, however, with the supposition that the potency of the inoculum was about the same throughout the entire period in which it was employed (Table I), although there can be no direct demonstration.

DISCUSSION

The most convincing method of showing active immunity to an infectious agent is to challenge recovered subjects with potent inocula. The number of subjects needed depends upon the attack rates in the experimental and control groups. In this investigation only five recovered volunteers could be brought together for reinoculation. Since none developed gastroenteritis after challenge with a dose of fecal supernate capable of infecting most of the volunteers exposed to it for the first time, however, it is logical to conclude that the experimental disease suffered by the five men approximately a year before had conferred substantial immunity that persisted during the interim.

This conclusion is at odds with the tentative interpretation of a previous test of duration of immunity in two other men (5). It was stated then that two subjects reinoculated approximately 10 months after an induced attack experienced a second bout of the disease, and it was suggested that immunity had diminished during that time. While the conflicting results may be due to the variation in the immune response of individuals to be expected in this as in other infections, re-examination of the clinical records of these two volunteers leads to another explanation. In the light of experience with the experimental disease gained since then, it is felt that one of the two men may not have had a definite illness following his first inoculation; the other one did, but displayed only borderline signs after reinoculation. The criteria for diagnosis in the present study were more strict, and no such doubts cloud the clinical findings on which the analyses presented here are founded.

Excluding these two questionable cases, a total of 21 persons recovered from a prior experimental illness have now been reinoculated with the Marcy agent and all were resistant to reinfection. Kojima and his co-workers (13) had almost identical results with a strain of afebrile nonbacterial gastroenteritis from Niigata Prefecture, Japan; each of 22 recovered volunteers whose immunity was similarly checked remained well following reinoculation. In the United States it has not yet been possible to carry out reciprocal tests of active immunity with two or more different strains of the afebrile type, as has been done with the afebrile and febrile types (1). Recent Japanese experiments indicate that infection with the Marcy strain may give immunity to one of the Japanese strains (14), but the full results are not available.

This study was conducted with a limited number of volunteers at a time when little inoculum known to be safe for human experimentation was on hand. It was hoped that by combining appropriate experiments a maximum amount of information about immunity in Marcy gastroenteritis could be gathered. This was accordingly done when men volunteered and opportunities arose, and was the reason that serum was mixed with the fecal inoculum for Inoculations 8 and 9 (Table I). Kojima and his co-workers (13) had failed to demonstrate neutralization of their Niigata strain by 2- to 3-week convalescent-phase sera from recovered subjects, and it was suspected that this might also be the case with the Marcy strain. For Inoculations 8 and 9 of our study, vials of supernate were withdrawn from the dry ice box, thawed, pooled, and mixed. To an appropriate volume of pooled supernate was added an equal volume of pooled acute-phase or convalescent-

phase serum respectively. The mixtures were held in an ice bath for an hour; it was feared that at a higher temperature the Marcy agent might be inactivated, experience having been restricted to experiments in which the inocula were kept cold. Under these conditions the results were like those of Kojima and his co-workers (13); there was no apparent neutralization of the Marcy agent by either pool. The information is of limited value since antigen-antibody combination may not have taken place at the temperature of melting ice, or if it did, dissociation of the complex may have ensued in the gastrointestinal tract. The additional procedure did not interfere, however, with use of the results to determine potency of the inoculum.

At present neutralization tests in orally inoculated volunteers seem impracticable for demonstrating humoral immunity or relating the incitant of a nonbacterial gastroenteritis outbreak to either the Marcy or the Niigata agents. Attempts to detect Marcy antigen in complement-fixation tests with treated fecal supernates or inoculated tissue cultures have also been unproductive (15). So far the incitants of nonbacterial gastroenteritis can be studied only in connection with clinical illness of humans and investigation is hampered by restriction to transmission experiments in volunteers.

The data presented here suggest that the agents, presumably viruses, that may be responsible for the periodic recurrences of diarrheal nonbacterial gastroenteritis that have been observed in the Northeastern United States (2, 3, 7, 8) are diverse. Whether different outbreaks are caused by unrelated viruses, by a family of viruses, or by antigenically different strains of a single virus is a subject for future investigation. The results of this study may be useful in approaching the problem.

SUMMARY

A study was made of the persistence of immunity in five human volunteers following infection induced by oral inoculation of the Marcy strain of afebrile nonbacterial gastroenteritis. Active immunity was tested by feeding an aliquot of an inoculum demonstrated to be highly infectious. Of the five recovered volunteers, four were brought together for the reinoculation experiment 15 months after onset of the initial experimental illness; the fifth had experienced his induced attack 9-½ months before. None of the five had a second attack, indicating that immunity had persisted during the interim. The results support the possibility that yearly recurrences of nonbacterial diarrheal disease may be due to separate nonbacterial agents, presumably viruses, or to antigenically different strains of the same agent.

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