Loss of Immunologic Reactivities of Australia Antigen After Incubation with Bacteria (37016)

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Australia antigen is a human serum antigen which is closely associated with the infectious agent of hepatitis (1–3). The antigen develops in the blood of patients during the incubation period and illness of acute viral hepatitis (4). Blood containing Australia antigen is capable of transmitting the disease (5).

In the course of studies on the effect of collagenase on Australia antigen, a serum was observed to lose its Australia antigen reactivity when incubated at 37°. *Pseudomonas aeruginosa* was isolated from this serum and it was found that incubation with this isolate reproducibly destroyed Australia antigen reactivity in the immunodiffusion test.

Materials and Methods. Australia antigen was detected using the immunodiffusion method employing a 7 hole pattern cut in 1.1% agarose in pH 8.2 Veronal buffer (6, 7). The sera which contained Australia antigen used in these studies were from patients with acute viral hepatitis and Down's syndrome. Trypticase soy broth and trypticase soy agar (Difco) were used for cultivating the bacteria at 37°. Bacteria were identified at the Jeanes Hospital Laboratory using the four part differential system (Kliglers iron agar, Simmons citrate agar, SIM medium and urea agar). Sterile filtrates were produced by passing cultures through a 1.2 μm Millipore filter and then a 0.45 μm filter.

Experiments and Results. A. The effect of temperature on the action of P. aeruginosa. The following experiment demonstrates the inactivating capacity of the cultures and shows that the loss of Australia antigen reactivity upon incubation with *P. aeruginosa* is temperature dependent. Three replicate vials containing 0.2 ml of 10 different sera which contained Australia antigen were incubated at 37°, 25° and in the refrigerator at 3–4° with 1 drop of a 1 day broth culture of *P. aeruginosa*. As a control, an additional set of the same 10 sera was incubated with 1 drop of sterile trypticase soy broth at each temperature. These 60 tubes were sampled at different times and tested for Australia antigen by immunodiffusion.

The number of sera in which Australia antigen could be detected after incubation with *P. aeruginosa* at different temperatures is indicated in Table I. At 37° most of the sera had lost their reactivity by the third day; only 2 of the 10 original positive sera remained positive on Day 3. In contrast, all of the specimens kept at 25° and in the refrigerator were still positive. By 7 days 4 of the 25° and all but one of the 37° specimens had been inactivated but the refrigerated samples were all still reactive. However, by the 20th day 3 sera kept in the refrigerator and 4 kept at 25° had lost reactivity. All of the samples at 37° were negative on Day 20. The trypticase soy broth plus Australia antigen serum controls were stable at all temperatures; Australia antigen was detectable at all times, with the exception of one control serum maintained at 37° for 20 days. The Australia antigen in this specimen may have been unstable at 37° or it may have been contaminated with bacteria during the experimental period. The loss of reactivity is clearly temperature dependent.

B. Possible mechanisms. Five possibilities arise concerning the mechanism of the loss of the Australia antigen reactivity in Australia antigen positive sera incubated with *P. aeruginosa*: (a) Substances produced in *P. aeruginosa* cultures may clog the agar pores

TABLE I. Number of Sera Remaining Positive for Australia antigen by Immunodiffusion After Incubation with *P. aeruginosa* at Different Temperatures.

Incubation (°)	Day: 1		3		7		20	
	TS[a]	Bact[b]	TS	Bact	TS	Bact	TS	Bact
37	10	10	10	2	10	1	6[c]	0
25	10	10	10	10	10	6	10	6
3–4	10	10	10	10	10	10	10	7

[a] TS = trypticase soy broth plus Australia antigen.

[b] Bact = Australia antigen plus *P. aeruginosa*.

[c] Three were not tested because of insufficient quantity; 1 was negative.

around the immunodiffusion well and prevent the diffusion of the Australia antigen out of the well. (b) Proteolytic enzymes released during *P. aeruginosa* growth may diffuse into the agar gel and destroy the reactivity of the anti-Australia antigen antibody which migrates from the center well. (c) Some nonenzymatic substance produced during growth may interfere with the antibody–antigen reaction. (d) The incubation of Australia antigen with *P. aeruginosa* may result in the adsorption of the antigenically reactive material to bacteria thus preventing it from migrating in the gel because the pores in the agar are too small to permit the passage of bacteria. (e) Finally, exoenzymes released into the growth medium may degrade the antigenic determinants of Australia antigen.

The following experiment was done to attempt to distinguish between these possibilities.

Three immunodiffusion patterns were prepared A, B and C (Fig. 1). The first pattern (A) contained Australia antigen controls in top and bottom wells plus two sera which had been converted from Australia antigen positive to Australia antigen negative by incubation with *P. aeruginosa* at 37° for 4 days (wells 1 and 2) and two Australia antigen negative sera which also had been incubated with *P. aeruginosa* in the same way (wells 3 and 4). Only the control Australia antigen (not reacted with *P. aeruginosa*) in the top and bottom wells of pattern A reacted with the anti-Australia antigen. Pattern B also contains control Australia antigen (top and bottom wells) and the same four sera which had been incubated with bacteria. Australia antigen was added to the 4 test wells and was allowed to be absorbed into the gel before the bacterially treated sera were added. Pattern C is the same except the Australia antigen was added after the treated sera. Precipitin lines occurred between the center well and all wells to which Australia antigen was added. This result indicates that suggestions (a), (b) and (c) are unlikely explanations for the loss of reactivity. The wells were not completely clogged by material generated during the incubation of serum with bacteria. If this were the case the Australia antigen added after the culture would not have been able to diffuse through the agar to form the precipitin band. Since this band was formed in pattern C this explanation for the loss of reactivity is eliminated. This same experiment also excludes the possibility that the immunodiffusion reaction between the antigen and antibody was prevented by exoenzymes or other materials generated during incubation. The reaction occurred regardless of whether the culture material was added before or after the antigen. Therefore, there was no interfering material in these preparations. The most plausible remaining explanations for the disappearance of Australia antigen reactivity after incubation with *P. aeruginosa* are (d) and (e). That is that the antigen may be adsorbed by the bacteria and thus prevented from migrating (d), or the immunoreactivity of Australia antigen is destroyed by substances produced during *P. aeruginosa* growth (e).

C. The effect of P. aeruginosa *culture filtrates on Australia antigen.* If exoenzymes or other soluble substances released during growth destroy the Australia antigen, filtrates

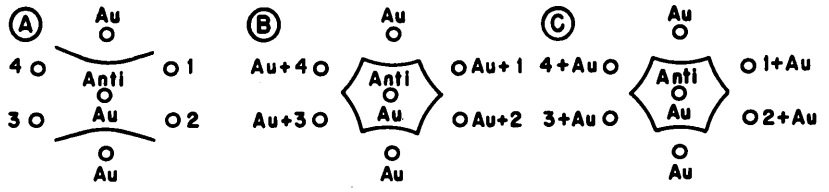

FIG. 1. Immunodiffusion results on sera incubated with *P. aeruginosa* and on the same sera preceded by and followed by Au (1) in the agar pattern: (Sera 1 and 2) Au (+) *P. aeruginosa* $\longrightarrow$ Au (—); (Sera 3 and 4) Au (—) *P. aeruginosa* $\longrightarrow$ Au (—). Pattern A contains control Australia antigen in the top and bottom wells. Wells 1 and 2 contain sera which gave a positive reaction for Australia antigen before incubation with bacteria. Wells 3 and 4 contain sera which gave a negative reaction for Australia antigen before incubation with bacteria. The only positive Australia antigen reactions were the control reactions. The treated sera were negative. Pattern B also contains control Australia antigen and the same 4 sera which had been incubated with bacteria. Australia antigen was added to the 4 test wells and adsorbed before the treated sera was added. Pattern C contains all the same materials as Pattern B. The only difference is that the Australia antigen was added after the treated sera. There were no substances in the sera which had been incubated with bacteria which could interfere with the formation of an Australia antigen immune precipitate band.

of the cultures should be equally effective in degrading the antigen. On the other hand, if Australia antigen is adsorbed by the bacteria, the presence of the bacteria would be required and filtrates should be ineffective.

In order to distinguish between these possibilities the following tests were made: Six 4.5 ml tubes of trypticase soy broth were inoculated with 0.5 ml of a 1 day culture of *P. aeruginosa*. At that time and daily for 5 successive days, one of the replicate culture tubes was examined in the following way.

The number of bacteria was determined each day by streaking 0.01 ml of 10-fold dilutions on trypticase soy agar plates and counting the resulting colonies. Then the cultures were filtered through a 1.2 μm prefilter and a 0.45 μm filter. One drop of each of these filtrates was added to trypticase soy broth tubes to test for the presence of bacteria and the remaining filtrate was frozen. Six tubes were prepared with these sterile filtrates. They consisted of 0.5 ml sterile serum from a patient with acute viral hepatitis which contained Australia antigen plus 0.5 ml of a particular filtrate or sterile trypticase soy broth. The pH of these mixtures was determined and they were incubated at 37° and tested for Australia antigen by immunodiffusion at the time they were mixed, and daily thereafter for 14 days. Each tube was tested daily for sterility by adding 1 drop to a trypticase soy broth tube. The results of this experiment are reported in Table II.

The filtrates prepared at the start of the experiment and those prepared at 24, 96 and 120 hr did not affect the Australia antigen. The filtrates prepared at 48 and 72 hr did destroy the reactivity of the antigen. The pH of the reaction mixtures varied between 6.8 and 7.2 and all incubation mixtures remained sterile during the period of observation. These results indicate that the Australia antigen immunoreactivity is destroyed by soluble substances which are produced during growth by the bacterial cultures between 48 and 72 hr. Absorption does not appear to be a factor since the presence of bacteria is not required for the destruction of Australia antigen.

D. The effect of heat on bacterial filtrates. The next experiment was designed to test whether active filtrates are heat sensitive. An active filtrate from a *P. aeruginosa* culture was dispensed into two glass tubes. One tube was incubated (100° for 10 min) in a water bath; the other was maintained at room temperature. An amount of serum containing Australia antigen equal to the filtrate was added to each tube. A control tube with the same amount of serum containing Australia antigen plus sterile trypticase soy broth instead of filtrate was also included. All were immediately retested for Australia antigen and sterility, and then incubated for 5 days

TABLE II. The Digestion of Australia Antigen Using Sterile Filtrates from *P. aeruginosa* Cultures.

Age of culture (hr)	No. of bacteria	pH of reaction	Time required[a] (days)
0	23×10^7	7.0	—
24	5.9×10^9	6.8	—
48	3.9×10^8	6.8	4
72	2.1×10^8	7.0	7
96	1.3×10^7	7.0	—
120	1.5×10^7	7.2	—

[a] Time required by the sterile filtrates to destroy the reactivity of Australia antigen when they are incubated together at 37°.

at 37°. On the fifth day and daily thereafter they were tested for Australia antigen and sterility. At the time the experiment was started all three preparations were sterile and positive for Australia antigen. This was also true on the fifth and sixth days. However, on Day 7 all preparations were still sterile but the Australia antigen incubated with the unheated bacterial filtrate failed to react in the immunodiffusion test.

This indicates that the soluble substance (or substances) released into the medium by *P. aeruginosa* which is responsible for the destruction of Australia antigen is also heat labile.

All of these results are compatible with the explanation that *P. aeruginosa* releases a soluble exoenzyme(s) which is heat labile and which destroys the immunological reactivity of Australia antigen.

E. The effect of fecal isolates on Australia antigen. We next wanted to determine if bacteria isolated from the human intestine would have the same effect as *P. aeruginosa* [*Pseudomonas aeruginosa* is said to occur in only 1 of 10 stool specimens (8).] We therefore isolated two bacterial species from feces on tripticase soy agar under aerobic conditions. These isolates were identified as *Escherichia coli* and *Staphylococcus aureus*. Cultures of these isolates were prepared and tested for their ability to alter Australia antigen. After incubation at 37° and daily testing for 9 days, it was found that *E. coli* destroyed Australia antigen in 5 days while *S. aureus* did not affect it during the 9 day period.

Filtrates of cultures of *E. coli* were made daily. They were tested for the ability to destroy Australia antigen in the following way:

Replicate cultures of *E. coli* were prepared by inoculating 0.5 ml of a 1 day culture into 4.5 ml of trypticase soy broth. At the time of preparation and daily for 4 days 0.1 ml from one culture was inoculated into 0.5 ml of sterile serum containing Australia antigen and the remaining culture filtered. One half milliliter of this filtrate was mixed with 0.5 ml of sterile serum containing Australia antigen. These preparations were tested at once and daily for 14 days for both sterility and Australia antigen. All filtrates remained sterile throughout the test period. Results of this experiment are presented in Table III. All serum cultures inoculated with bacteria lost their Australia antigen reactivity in from 4 to 7 days. Filtrates from Days 2 and 4 destroyed Australia antigen during the period of observation. These results indicate that *E. coli*, like *P. aeruginosa*, releases filterable substances which abolish Australia antigen reactivity.

It was determined, using the procedures described above for *P. aeruginosa* that these substances are destroyed by heating to 100° for 10 min. This implies that the destruction of Australia antigen by *E. coli* may be accomplished by heat-sensitive enzymes, as appeared to be the case for *P. aeruginosa*.

F. The effect of prefreezing on the action of bacteria. The action of bacteria on Australia antigen in serum which requires shipping may be a very serious practical problem under some field conditions. An experiment was designed to determine whether subjecting a contaminated serum specimen which

TABLE III. The Destruction of Australia antigen by Bacteria and Sterile Filtrates from the Same *E. coli* Cultures of Different Ages.

Age[a] of culture	0	1	2	3	4
Time[a] required for *E. coli* to destroy Australia antigen	4	5	4	7	4
Time[a] required for filtrate to destroy Australia antigen	—	—	7	—	3

[a] In days.

contained Australia antigen to freezing and thawing would effect subsequent digestion of Australia antigen in those samples.

Sterile serum which contained Australia antigen was divided into five 0.5 ml aliquots. Two were inoculated with 1 drop of a 1 day culture of *E. coli* and two were inoculated with 1 drop of a 1 day culture of *P. aeruginosa*. The fifth was inoculated with trypticase soy broth as a control. One of each of the bacterial preparations was frozen at —20° for 24 hr and the other was kept at 37°. The preparations were thawed and all preparations were then kept at 37° and tested daily by immunodiffusion for the presence of Australia antigen. Both of the *E. coli* preparations were negative for Australia antigen after 4 days at 37°. The *P. aeruginosa* preparation which had been frozen lost reactivity 1 day later than the one kept at 37°. These two lost reactivity at 5 and 6 days, respectively. The trypticase soy control remained positive for Australia antigen throughout the experiment. Short-term freezing of contaminated sera has no significant effect on the subsequent destruction of Australia antigen by *P. aeruginosa* or *E. coli* at 37°.

Discussion. The destruction of Australia antigen can be reproducibly accomplished with sterile filtrates of either *P. aeruginosa* or *E. coli*. This activity is destroyed by heating at 100° for 10 min. Both of these results are compatible with the hypothesis that the destruction of Australia antigen is caused by bacterial exoenzymes.

This finding that common bacteria can convert Australia antigen positive serum to an Australia antigen negative serum has obvious application to the storage and shipping of specimens to be used for testing for Australia antigen. Sterile samples of serum containing Australia antigen are remarkably stable, remaining reactive at room temperature for more than 1 yr (9). However, because of possible loss of reactivity due to bacterial contamination it is recommended that samples be kept frozen whenever possible and not allowed to remain at ambient temperatures for long periods of time. Samples should be shipped by the fastest means possible and should be frozen when practical. Prefreezing a sample does not substantially alter the effect of the two bacteria studied when the contaminated samples are returned to 37°. It is probable that bacteria other than those included in this study may also be able to degrade Australia antigen and produce false negative results. In addition, the data presented in this communication suggest the following hypotheses which could have important epidemiological implications.

Bacterial degradation of Australia antigen may occur in the intestine. Australia antigen has been identified in the bile (10). Australia antigen is sometimes present in the feces of Australia antigen positive hepatitis patients (11, 12). However, this has been demonstrated with some irregularity (13). This may be because although the Australia antigen enters the gastrointestinal tract it can be degraded during its passage through it.

If the Australia antigen particle itself is infectious, any alteration of the particle must be important epidemiologically. The observed alteration of the immunological identity of the particle may or may not be accompanied by a change in infectivity. It is possible that infectious Australia antigen is released into the intestine in the bile and immunologically altered by enzymes in the intestine. The feces may then emerge carrying particles which are no longer identifiable as Australia antigen but which remain infectious.

The authors thank Dr. Gordon D. Benson of the Jefferson Medical College of the Thomas Jefferson University Hospital in Philadelphia for providing them with many of the Australia antigen samples required by these studies. This work was supported by U.S. Public Health Service Grants CA-06551, CA-06927 and RR-05539 from the National Institutes of Health, and by an appropriation from the Commonwealth of Pennsylvania.

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Received Sept. 29, 1972. P.S.E.B.M., 1973, Vol. 142.