# Specificities of Human Antibodies to Australia Antigen[1] (34833)

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Australia antigen [Au(1)] was first detected in the serum of an Australian aborigine using as an antiserum a precipitating antibody which developed in the serum of a transfused patient (1, 2). It has since been shown that this antigen is a particle intimately related with or a causative agent of viral hepatitis (3–7). The studies from our laboratories supporting this hypothesis have been reviewed (8–14) and confirmation of these findings has now come from several other laboratories (see, for example 15–19).

The initial studies on Australia antigen were done using human antiserum (2). Later, antisera were produced in rabbits by immunization with human serum containing Australia antigen and subsequent absorption with serum without Au(1) (20). In the early studies it was recognized that there were minor differences between the human antisera. This was shown by rare examples of spurring in Ouchterlony immunodiffusion experiments and by differences in the pattern of reactions of different antisera against a standard panel of sera containing Australia antigen. An antiserum with clearly different specificities was produced by Levene and Blumberg (21) by immunizing a rabbit with the serum of an individual which gave what appeared to be a partial reaction with the original anti-Au(1) serum. This "new" antiserum was termed anti-Au(2). It was shown that anti-Au(1) had at least two specificities (a, c) and anti-Au(2) at least two specificities (a, b). One of these was common to both antisera, and each antiserum had a specificity that the other did not have. Since these initial findings antisera have also been found in individuals who have not received transfusions but presumably became sensitized to Australia antigen by other means.

In the present paper we describe studies designed to demonstrate differences between human antisera against Australia antigen. This is done to further define the Australia antigen system and to attempt to identify other specificities of the antigen which could relate to viruses responsible for other forms of hepatitis and related diseases. We found that all the antisera had specificities in common, but by the selection of appropriate antigens it was possible to demonstrate some differences between many of the antisera.

*Materials and Methods.* A series of techniques have been used to demonstrate differences between antisera. They follow in part the methods used previously in distinguishing multiple specificities found in the Ag lipoprotein system (22).

*Antisera and antigens.* In order to increase the probability of identifying different kinds of antisera, sera containing antibodies to Australia antigen from different parts of the world were obtained and these were compared using panels of antigens selected both from the immediate environment of the individual with the antiserum and from the foreign environments from which the other antisera originated. Since it is possible that (1) different antigenic varieties of organisms or different modes of an organism might develop and thrive under different ecological conditions, and (2) individuals living in different parts of the world might develop different antibody responses, this appeared to be a logical technique to use in looking for major differences in antisera specificities. Three of

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TABLE I. Characteristics of Individuals in Whom Antibodies Were Found.

| Geographic location | Population | Abbreviation | ICR number | Diagnosis | Transfusions | Sex | Age | Date collected |
|---|---|---|---|---|---|---|---|---|
| Mactan Island, Cebu, Philippines | Filipino | Cebu | CO-34345 | Lepromatous leprosy | No | F | 41 | 11–14–67 |
| Frobisher Bay Baffin Island N.W.T., Canada | Eskimo | F. B. | CO-44064 | Apparently normal | No | F | 24 | 4– 4–68 |
| Hong Kong, China | Chinese | H. K. | CO-51653 | Lepromatous leprosy | No | M | 28 | 1–29–69 |
| Philadelphia, Pennsylvania | U.S. white | Phila. A | CO-41177 | Aplastic anemia | Many | F | | 10– 1–68 |
| Philadelphia, Pennsylvania | U.S. white | Phila. B | CO-41938 | Esophageal ulcer | Many | M | 52 | 2–11–68 |
| Philadelphia, Pennsylvania | U.S. white | Phila. C | CO-41683 | Chronic renal dis. | Many | M | | 1–22–68 |
| Philadelphia, Pennsylvania | U.S. white | Phila. D | CO-41222 | Thalassemia | Many | F | 10 | 9–23–68 |
| Philadelphia, Pennsylvania | U.S. white | Phila. E | CO-45374 | Mental retard. | Transfused at birth, not since | M | 18 | 1–27–69 |
| Surinam, South America | — | Surinam | CO-45875 | Apparently normal | No | F | 11 | 6–12–68 |

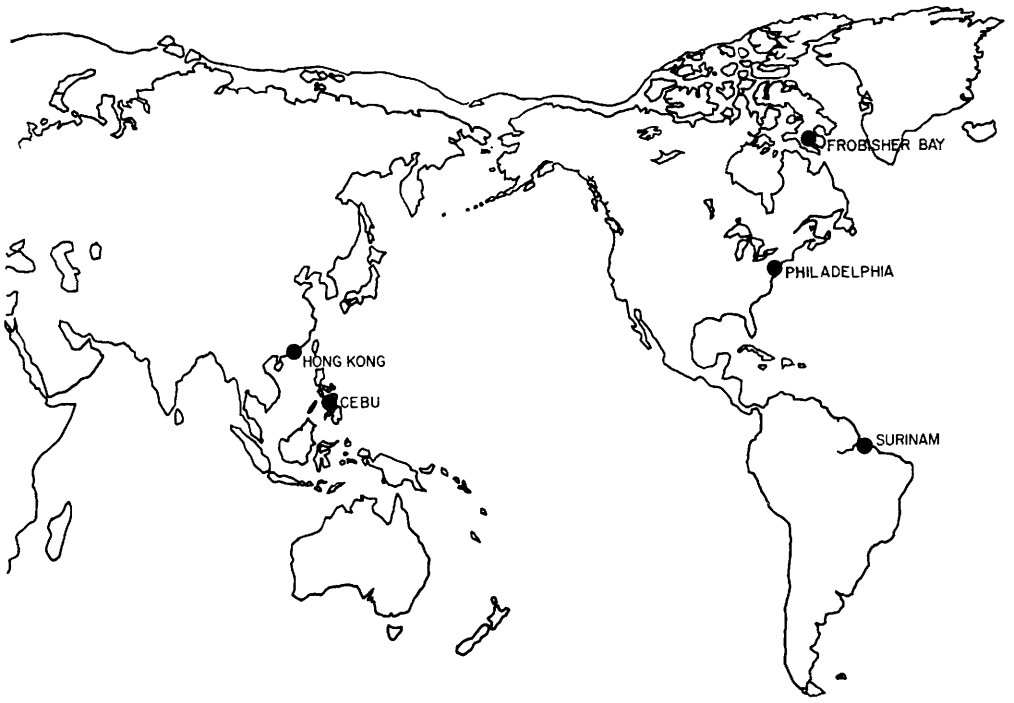


FIG. 1. Locations at which sera were collected. Frobisher Bay, Baffin Island, N.W.T. Canada; Hong Kong, B.C.C., China; Cebu, Philippines; Philadelphia, Pennsylvania; Surinam, South America.

the antisera were from individuals who had not received transfusions. One serum was from an 18-year-old boy who received a single transfusion at birth. The remaining five were from multiply transfused patients. The characteristics of the individuals in whom the antibodies were found are shown in Table I, and the location where they were collected in Fig. 1.

The antigen panels consisted of apparently normal people and patients from Cebu, Frobisher Bay, Hong Kong, Surinam, and Philadelphia. Most of these sera had given a positive reaction with a Philadelphia A antiserum, a "standard" human anti-Au(1) which had been used in many of our previous studies (see below). This antiserum and one similar to it have also been compared to many of the antisera used in other laboratories [see, for example, Okochi (17), Gocke (18), Holland (23), and Prince (19)]. We also included sera in the panel which did not react with Philadelphia A. The total antigen panel consisted of 55 different sera (Table II).

Two complementary methods were used to determine if there were differences between the antisera, (1) the population method and (2) the immunologic method. The population method (described below) was used first. From this, certain sera could not be distinguished from each other, and were classified in the same category (Philadelphia A), while others were shown to be different and formed two additional categories. All the antisera were then tested using the immunologic method (also described below).

*The population method.* Each antiserum was tested against all sera in the antigen panel. The comparisons of the antisera with each other were then arranged as shown in Fig. 2. In this the number of individuals positive with both antisera, negative with both antisera, and positive with one but not the other are recorded in 2 × 2 tables. If the two antisera being compared (*e.g.* antiserum I, antiserum II) are identical in specificity and strength, then all antigens (Table II) positive with antiserum I would be positive for antisera II and all negative with I would

TABLE II. Antigen Panel.

| Location | Population | Diagnosis | | Number of samples |
|---|---|---|---|---|
| Cebu | Filipino | Lepromatous or tuberculoid leprosy | 5 | |
| | | Normal | 5 | 10 |
| Frobisher Bay | Eskimo | Not given | | 5 |
| Hong Kong | Chinese | Lepromatous or tuberculoid leprosy | 4 | |
| | | Miscellaneous | 6 | 10 |
| Philadelphia | U.S. White | Down's syndrome | 10 | |
| | | Hepatitis | 7 | |
| | | Chronic renal dis. | 3 | 20 |
| Surinam | Japanese, Djuka, Hindustani | Not given | | 10 |
| TOTAL | | | | 55 |

be negative with II (array A, Fig. 2). If the antisera were identical in specificity but II was of greater strength than I, then some of the panel sera would be positive with II but negative with I (array B, Fig. 2). Alternatively, if I and II were identical, but I was stronger than II, the distribution would follow array C. For arrays B and C, one could state that the antisera could not be distinguished from each other with certainty and they could be tentatively classified as the same. If all combinations of reactions are seen (array D), then this implies that both antisera are multispecific. Further, it could also mean that there are one or more specificities which antisera I and II do not have in common or that antiserum I has one or more specificities at greater strength than the corresponding specificities in antiserum II; and antiserum II in turn, has another specificity or specificities, stronger than corresponding specificities in antiserum I. These relationships were analyzed by the chi-square distribution and the calculation of contingency coefficients.

*The immunologic method.* In the next series of experiments the antisera were compared to each other using selected antigens in micro-double-diffusion plates as described by Wadsworth (24) and modified by Raunio (25). If two antisera were different in respect of one or more specificities then this would be reflected in the development of spurs between the appropriate wells. A limitation of comparative double-diffusion analysis is the failure of spurs to form under some conditions even though the antisera have different specificities. This has been discussed by Ouchterlony (26). However, the demonstration of spurs makes it highly probable that there are different specificities providing that the antisera are not greatly different in strength. Hence, in this method the identification of spurs gives positive information on differences between the antisera but the failure to find spurs is not informative.

*Results and Discussion.* From the examination of the arrays of Fig. 3, Philadelphia A, Philadelphia C, Philadelphia D, Cebu, Frobisher Bay, and Hong Kong could not be

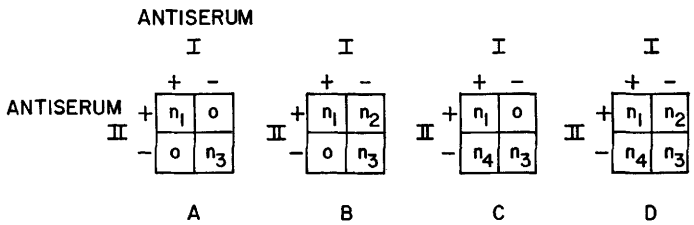


FIG. 2. Comparison of antisera using 2 × 2 tables. Four possible arrays are illustrated and these are explained in the text.

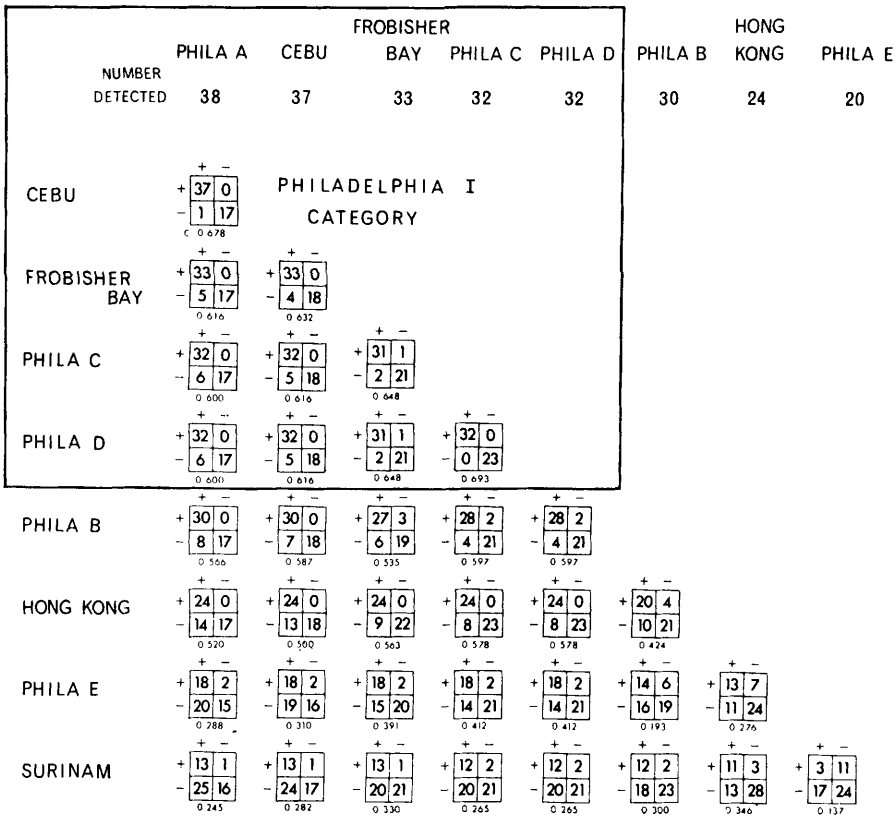


FIG. 3. Comparison of antisera using the population method. The 2 × 2 tables are arranged so that each antiserum is compared with every other. Antisera are ranked in order of frequency of reactions of panel. C = contingency coefficient. The results are explained in the text.

distinguished from each other, *i.e.*, they conformed to either array A, array B, or array C of Fig. 2. Philadelphia B could not be distinguished from Philadelphia A but may be different from the other antisera just listed (*i.e.*, array D).

Both Philadelphia E and Surinam, when compared to all other antisera and to each other, conformed to array D (Fig. 2); that is, they are either different from each other and all the others or (as discussed in the Methods) they have two or more similar specificities whch are present in different strengths.

To quantitate the degree of association between the antisera, contingency coefficients were calculated for each of the 2 × 2 comparisons of Fig. 3 and ranked in order (Table III). The highest associations are between Philadelphia A, Philadelphia C, Philadelphia D, Cebu, and Frobisher Bay (contingency coefficients 0.600 to 0.693); we shall now refer to these antisera as category Philadelphia I (Table IV). All other antisera (excepting Philadelphia B) showed a lesser degree of association with either the members of Philadelphia I, or with each other. Philadelphia B falls into an intermediate position.

The results of the immunologic method are illustrated in Fig. 4. Members of Philadelphia I have two or three bands when tested against appropriate sera containing Australia antigen, whereas Philadelphia E and Surinam antisera had only a single line (Figs. 4 and 5). This could mean that members of Philadelphia I have multiple specificities, whereas Philadelphia E and Surinam have fewer specificities and could be monospecific. When members of Philadelphia I were compared by the immunodiffusion method, the three or

TABLE III. The Antisera Comparisons Ranked in Order of Contingency Coefficients Calculated for Each 2 × 2 Table in Fig. 3.

| Antisera | Contingency coefficient | Antisera | Contingency coefficient |
|---|---|---|---|
| Phila. C –Phila. D | 0.693 | Phila. E–Phila. C | 0.412 |
| Phila. A –Cebu | 0.678 | Phila. E–Phila. D | 0.412 |
| Phila. C –F. B. | 0.648 | Phila. E–F. B. | 0.391 |
| Phila. D –F. B. | 0.648 | Surinam–Hong Kong | 0.346 |
| Cebu –F. B. | 0.632 | Surinam–F. B. | 0.330 |
| Cebu –Phila. C | 0.616 | Phila. E–Cebu | 0.310 |
| Cebu –Phila. D | 0.616 | Surinam–Phila. B | 0.300 |
| Phila. A –F. B. | 0.616 | Phila. E–Phila. A | 0.288 |
| Phila. C –Phila. A | 0.600 | Surinam–Cebu | 0.282 |
| Phila. D –Phila. A | 0.600 | Phila. E–Hong Kong | 0.276 |
| Phila. C –Phila. B | 0.597 | Surinam–Phila. C | 0.265 |
| Phila. D –Phila. B | 0.597 | Surinam–Phila. D | 0.265 |
| Cebu –Phila. B | 0.587 | Surinam–Phila. A | 0.245 |
| Phila. C –Hong Kong | 0.578 | Phila. B–Phila. E | 0.193 |
| Phila. D –Hong Kong | 0.578 | Surinam–Phila. E | 0.137 |
| Phila. A –Phila. B | 0.566 | | |
| F. B. –Hong Kong | 0.563 | | |
| F. B. –Phila. B | 0.535 | | |
| Phila. A –Hong Kong | 0.520 | | |
| Cebu –Hong Kong | 0.500 | | |
| Hong Kong–Phila. B | 0.424 | | |

two bands were usually continuous showing reaction of identity. Further, Philadelphia A antiserum appears to encompass all the specificities present in the members of Philadelphia I. Hong Kong antiserum, when tested against certain positive sera, showed an extra band which did not merge with the others (Fig. 4). Interestingly, this band showed reaction of identity with a single precipitin band which was found between Hong Kong antiserum and Philadelphia E antiserum (Fig. 4). This precipitin, which does not appear to have an immunologic cross reactivity with Australia antigen, has been tentatively designated Hong Kong precipitin and will be described in detail in a separate paper.

A spur was observed between Philadelphia A and Philadelphia E antisera. There was no spur or other deviations indicating differences between Philadelphia E and Surinam as might have been expected from the population studies. Ouchterlony (26) has stated that antisera with different specificities do not always show spurs or other formations indicating differences between these antisera. If an antigen particle carrying two or more determinants of separate specificities is used as an indicator for the comparison of the antisera, then a pattern resembling a typical reaction of identify may result.

We have to consider the possibility that the multiple precipitin bands which occur in double-diffusion plates are artifacts, *i.e.*, secondary precipitates of the Liesegang type (27). This possibility was studied in the case of the Philadelphia A antiserum which gave three precipitin bands with a serum containing Australia antigen, R.Mc. (CO 45292) and D.M. (CO 45396).

TABLE IV. Categories of Antisera Distinguished by a Combination of the Population and Immunologic Method.

| Philadelphia I category | Other distinctive antisera | | |
|---|---|---|---|
| Phila. A (anti-Au(1)) | Phila. E | Surinam | Hong Kong |
| Phila. C | | | |
| Phila. D | | | |
| Cebu | | | |
| Frobisher Bay Phila. B | | | |

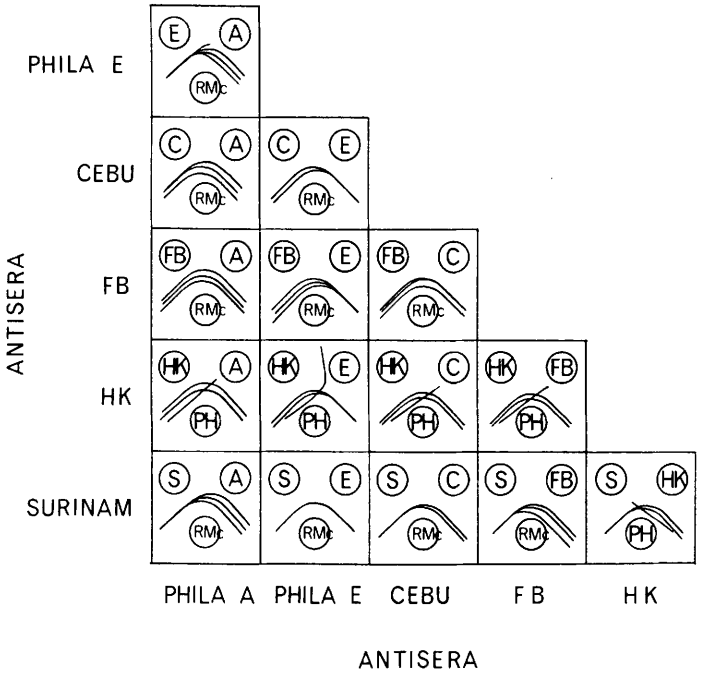


FIG. 4. Comparison of the antisera using the double-diffusion method. Serum containing Australia antigen (Au) was placed in the center wells of each of the patterns. Two different sera were used (R.Mc. and CO 45961). The antisera types are designated by letters: A = Philadelphia A; E = Philadelphia E; C = Cebu; F.B. = Frobisher Bay; H. K. = Hong Kong; S = Surinam.

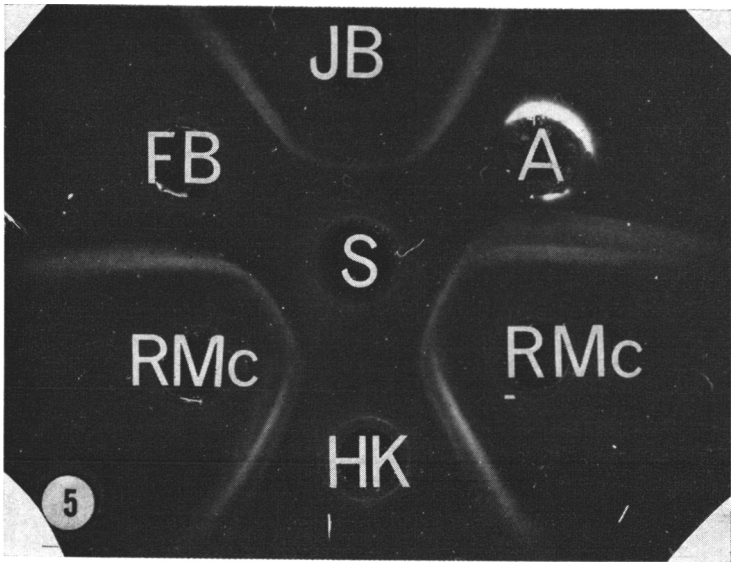


FIG. 5. Comparison of Surinam antiserum to the others. A single band is seen between Surinam antiserum (S) and positive samples J.B. and R.Mc. There are multiple bands between these antigens and Philadelphia A (A), Frobisher Bay (F.B.), and Hong Kong (H.K.) antisera.

1. Using the in-well absorption technique, Philadelphia A antiserum was absorbed with a serum J.B. (CO 40690), which gave only two precipitin bands with Philadelphia A. When subsequently tested by the micro-double-diffusion plate method, only one band

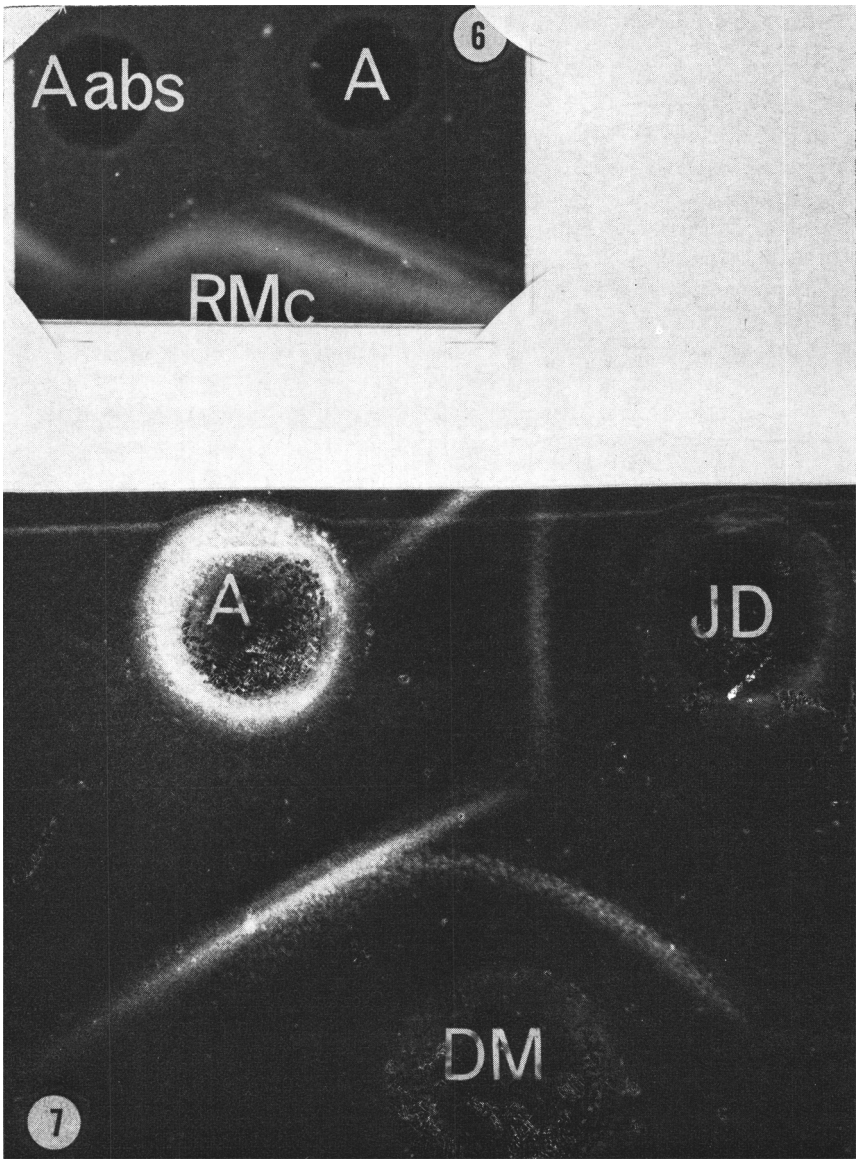


FIG. 6. Studies on multiple bands. Philadelphia A antiserum (A) gives three bands with serum R.Mc. Philadelphia A antiserum is absorbed with serum J.B. with which it reacts to give only two bands; this absorbed antiserum is designated A abs. It reacts with serum R.Mc. to give only a single band.

FIG. 7. Demonstration of the presence of one Australia antigen specificity and an antibody against another specificity of Australia antigen in the same serum sample. Philadelphia A antiserum (A) reacts with serum J.D. to give a single band, and with serum D.M. to give three bands. Serum J.D. also reacts with a single one of the specificities in serum D.M., but not the others. This suggests that serum 78 contains one of the antigenic specificities revealed by Philadelphia A, and also an antibody against another of these specificities.

occurred between the absorbed Philadelphia A antiserum and serum R. Mc. (Fig. 6).

2. A second serum J.D. (CO 45378) gave only one precipitin band with Philadelphia A. In addition, this sample reacted with serum D.M. (CO 45396), also giving a single precipitin band. This band was continuous with one of the three bands between Philadelphia A and serum D.M. CO 45396 (Fig. 7). This means that serum J.D. contains one of the antigenic specificities as well as antibody against another of the specificities; this is strong evidence that there are multiple specificities of Australia antigen.

3. Serum D.M. and Philadelphia A were diluted 2- and 4-fold and each dilution of antigen tested against each dilution of antiserum. The multiple bands were seen in every case. For these reasons we conclude that the multiple bands between Philadelphia A and sera D.M. and R.Mc. are not secondary precipitates but bands which represent multispecificities of the reactants involved.

All the data reported here support the hypothesis that there are multiple specificities of Australia antigen and that different specificities of antibody can form in different individuals. At least four categories of antisera have been shown. The existence of multiple, antigenic determinants has been shown for many particulate antigens and it would not be surprising if this should be the case for Australia antigen as well.

Most of the antisera fall within category Philadelphia I, and these include all the antisera from transfused patients (Table IV). It is antisera of this type which have been used extensively in the studies from our laboratory and recently from other laboratories. For example, the antisera used by Gocke (18), Holland (23), and Prince (19) and others have been compared to Philadelphia A and have been shown to have reactions of identity.

The selection of an appropriate nomenclature for these antisera is difficult since some of them are multispecific and complex. We assigned the designation anti-Au(1) to the first category of antisera we described anticipating that additional antisera might be found and knowing that the antisera contained more than one specificity. The term anti-Au(2) was assigned to the second kind of antiserum we studied; this was an antiserum produced by a unique rabbit immunization. Philadelphia A antiserum is the equivalent of anti-Au(1), and the other antisera in category Philadelphia I include the specificities encompassed by that designation. We do not propose to apply new number designations to the other antisera categories until they are further characterized. The unusual antisera (*i.e.*, Philadelphia E, Surinam, and Hong Kong) were all from patients who had not been transfused; the significance of this is not known.

*Summary.* Nine different human anti-Australia antigen antisera were compared to each other using population and immunologic methods. Six of the antisera fell into the same category (Philadelphia I) and included specificities detected by the previously described anti-Au(1) antisera. There were three antisera, each of which contained specificities different from those in the anti-Au(1) category. They are to be examined to see whether they identify forms of hepatitis or other conditions different from those detected by anti-Au(1).

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