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ANTIGENIC STRUCTURE OF *MYCOBACTERIUM LEPRAEMURIUM*

I. PRESENCE OF AVIAN TYPE α ANTIGEN IN STRAINS OF *M. LEPRAEMURIUM*

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SUMMARY Antigenic analyses were made of 8 strains of *M. lepraemurium* by Ouchterlony's immunodiffusion technique employing α and β antigens of *M. tuberculosis* and their specific antisera as indicators. It was found that crude extracts of all the test strains contain a cross-reacting material (*crm*) whose antigenic determinants are partially in common with α while no common antigen to β is detectable in them. Comparative analyses of the *crm* (designated as *Lepraemurium* α) with *Avian* and *P8* type α led to the tentative conclusion that the *Lepraemurium* α may be identical with *Avian* α antigen. The significance of this finding is discussed in terms of the serological typing of the organism in mycobacteria.

INTRODUCTION

The α and β antigens, which are major extracellular protein products of *M. tuberculosis*, have recently been shown to play a significant role as parameters in serological typing for mycobacterial classification (YONEDA, FUKUI and YAMANOUCHI, 1965). Thus, strains of mycobacteria can be classified by the distribution patterns of α and β antigens and also by the type of cross-reacting material (*crm*) with α antigen, of which two types (*Avian* and *P8* α) have so far been recognized. During the course of the present investigation, it was found that the crude cell extracts of two strains (Hawaii and Douglas) of *M. lepraemurium* tested contained no detectable β but a *crm* whose antigenic determinants were partially in common with those of α antigen of *M. tuberculosis*. Although this result was only preliminary and was on only two strains, we considered this finding very important as it was the first step in the elucidation of the immunochemical relationship between this species and other mycobacteria. Plans were therefore made in our two Departments for an investigation to confirm this observation and to see whether it was generally applicable to all available strains of *M. lepraemurium* and to see to which type the *crm* in murine leprosy bacilli belongs. It was also thought that the solution

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to these questions would elucidate the profile of the antigenic structure of murine leprosy bacilli and give information on the taxonomical position of the bacillus in terms of the serological typing of mycobacteria.

This paper reports the distribution pattern of α and β antigens in 8 strains of *M. lepraemurium* and results of comparative analyses of their cross-reacting materials (*crm*) with *Avian* and *P8* type α antigen.

MATERIALS AND METHODS

1. *Organisms*

All the strains of *M. lepraemurium* tested were from the stock collection of the Department of Leprology of this Institute. The names and sources of these strains are listed in Table 1.

2. *Collection and storage of the organisms*

A 0.1 ml aliquot of emulsion of leproma, diluted 1,000 times with physiological saline, was inoculated subcutaneously into the FL hybrid of C3H ♂ and CFI ♀ mice (KAWAGUCHI, 1959). Three to 5 months after the inoculation, the lepromas which had developed in the subcutaneous region were taken and from them murine leprosy bacilli were collected by low speed centrifugation using a slight modification of the method of MORI et al. (1961) and stored in a lyophylized state.

3. *Preparation of crude antigens of M. lepraemurium*

The lyophylized material from each strain was first mixed with roughly an equal volume of quartz powder and ground in a small mortar for several minutes in the cold. The resulting material was then mixed with roughly an equal volume of phosphate buffer (M/100, pH 7.0) containing 0.25 M sucrose. The mixture was then centrifuged at $12{,}000 \times g$ for 20 minutes and the resulting supernatant was again centrifuged at $105{,}000 \times g$ for 90 minutes in an ultracentrifuge, Spinco Model L. The clear portion of the resulting supernatant was dialyzed overnight against multiple changes of deionized water in the cold. The dialyzed material was finally lyophylized and stocked before use.

4. *Indicator antigens and antisera*

The highly purified preparations of α and β anti-

TABLE 1 *List of test strains of M. lepraemurium*

Strain	Isolated by	Received from
Hawaii	BADGER & FITE (1935) *National Inst. of Health Bull.* No. **173**, 77 (1940)	Dr. HANKS (1952)
Douglas	DOUGLAS (1922) *J. Path. Bact.* **45**, 739 (1937)	Dr. REES (1959)
Odessa	STEFANSKY (1903) *Z. für Bakt. Orig.* **33**, 481 (1903).	Dr. CHAUSSINAND (1957)
Kurume	URABE & MYAZAKI (1943) *Nippon Igaku* No. **3369**, 341 (1944)	Dr. NAKAMURA (1959)
Fukuoka	URABE (1940) Unpublished	Dr. URABE (1948)
Keishicho	UCHIDA (1921) *Tokyo Ijishinshi* **2252**, 1 (1921)	Dr. SATO (1953)
Kumamoto	OTAWARA & ICHIHARA (1931) *Tokyo Ijishinshi* **2729**, 8 (1931)	Dr. WATANABE (1941)
Osaka No. 1	NISHIMURA (1963) *La Lepro* **32**, 1 (1963)	

gens described previously (YONEDA and FUKUI, 1963; FUKUI, HIRAI, UCHIDA and YONEDA, 1965) were employed as indicator antigens. Unless otherwise specified, a concentration of 30 μg protein per ml of these antigens in M/15 phosphate buffer (pH 7.0) was used for agar gel diffusion analyses. Anti $-\alpha$ and $-\beta$ rabbit sera, which were used as indicator antisera, were prepared by immunizing rabbits with highly purified preparations of α and β antigens in incomplete Freund's adjuvants according to the schedule described previously (FUKUI, HIRAI, UCHIDA and YONEDA, 1965; YONEDA and FUKUI, 1961 a).

5. *Avian and P8 α antigens and their specific antisera*

The *Avian* and *P8* α antigens employed were obtained from concentrated unheated culture filtrates of the 4110 strain of *M. avium* and of the P8 strain of *M. kansasii*, provided by Dr. *E. Runyon*. These antigens were isolated and purified by a combination of ammonium sulfate fractionation, zone-electrophoresis and DEAE-cellulose column chromatography as described previously for the α antigen of *M. tuberculosis* (YONEDA and FUKUI, 1961 a, b; FUKUI and YONEDA, 1961; YONEDA and FUKUI, 1965). The antisera of *Avian* and *P8* α were prepared by immunizing rabbits with highly purified preparations of these antigens in incomplete Freund's adjuvant as described previously (YONEDA and FUKUI, 1961 a). In the present work, the first and booster dose injected was 50μg protein per rabbit respectively.

6. *Immunodiffusion test*

The double diffusion precipitation method in agar gel described by OUCHTERLONY (1948) was employed with a slight modification. Unless otherwise stated, the three wells in the Ouchterlony's plate employed for antigenic analyses of test strains were arranged in a regular triangular pattern and in these were put the test antigens, indicator antigens and antiserum respectively, so that the presence and immunochemical status of the common antigen with α or β in the test antigens could be examined. The results were usually read after standing the agar plates at room temperature for five to seven days.

RESULTS

1. *Distribution of α and β antigens in test strains of M. lepraemurium*

Experiments were made to see if all available strains of *M. lepraemurium* show a similar profile in the distribution pattern of α and β antigens to that previously observed with two strains (Hawaii and Douglas; YONEDA, FUKUI and YAMANOUCHI, 1965).

Immunodiffusion was carried out in Ouchterlony's plates employing crude antigens of 8 strains of *M. lepraemurium*, namely, Hawaii, Douglas, Odessa, Kurume, Fukuoka, Keishicho, Kumamoto and Osaka No. 1. The results are illustrated in Figs. 1a and 1b.

As can be seen from Fig. 1a, the test antigens of all strains reacted with anti $-\alpha$ serum each forming a single precipitation line which fused, with clear spur formation, with the single line formed between α antigen and its specific antiserum. On the other hand, as seen in Figure 1b, no precipitation line could be detected between these antigens and anti $-\beta$ serum. These results clearly indicate the absence of a common antigen to β, but show that there is *crm* with antigenic determinants partially in common with those of α antigen in all the antigens tested. For convenience, the *crm* found in these strains was designated as *Lepraemurium* α.

2. *Serological relation of Lepraemurium α to Avian and P8 α*

Cross-reacting materials (*crm*) with α antigen of *M. tuberculosis* are found to be distributed rather widely in various mycobacteria and yet they can be grouped into two distinct types (*Avian* and *P8* α) depending on the identity of their serological specificity (YONEDA, FUKUI and YAMANOUCHI, 1965). It was therefore of interest to know whether *Lepraemurium* α belongs one of these types. Thus, the serological specificity of *Lepraemurium* α was compared with that of *Avian* and *P8* α antigens by Ouchterlony's immunodiffusion method. In this experiment, purified preparations of *Avian* and *P8* α antigens and their specific antisera were used as indicators. A comparison of the agar precipitation patterns of *Lepraemurium* α and *P8* α is given in Fig. 2.

As seen in this figure, all the test antigens reacted with anti *P8* α serum forming a single precipitation line which fused, with clear spur formation, with the single line formed between *P8* α and its antiserum. This indicates that the antigen (*Lepraemurium* α) responsible for forming a precipitation line with anti *P8* α serum is a cross-reacting material (*crm*) with antigenic determinants partially in common with those of *P8* α.

Fig. 3 shows the results of a similar comparison by immunodiffusion of the test *Lepraemurium* antigens with *Avian* α.

It may be seen from the figure, that the patterns are entirely different from those obtained with *P8* α. Thus the single lines of the test antigens completely fuse with the single precipitation line formed between indicator antigen, *Avian* α, and anti *Avian* α serum, respectively. This clearly indicates that *Lepraemurium* α is indistinguishable by the present test from *Avian* α antigen in terms of its serological specificity.

DISCUSSION

It is now clear that strains belonging to the species, *M. lepraemurium*, all show a similar profile in terms of the distribution pattern of α and β antigens and they have no detectable β but a cross-reacting material (*Lepraemurium* α) with antigenic determinants partially in common with α antigen. Thus, the present results not only confirm our previous observations with two strains (Hawaii and Douglas) but also support the possibility that the antigenic profile described above may be a common feature of the strains belonging to the species, *M. lepraemurium*. As previously reported (YONEDA, FUKUI and YAMANOUCHI, 1965), strains of mycobacteria can be classified by the distribution pattern of their α and β, and, so far, they have been divided into four general serological groups, in which the 3rd group includes the strains characterized by the absence of β but presence of a *crm* whose antigenic determinants are partially in common with α antigen. Thus, it seems reasonable to put *M. lepraemurium* into the 3rd group to which several other species, such as *M. kansasii*, *M. avium* etc., are found to belong.

With the demonstration of the difference in antigenic specificity (YONEDA, FUKUI and YAMANOUCHI, 1965), it has recently been possible to divide the *crm* with α antigen into two types, namely, *Avian* and *P8* (*M. kansasii*) type α, and thus the 3rd group could be divided further into two subgroups (the Avian and P8 groups). The data (Figs. 3 and 4) presented here clearly show that, as far as the immunodiffusion test with anti *Avian* α and *P8* α sera is concerned, *Lepraemurium* α is different from *P8* α but indistinguishable from *Avian* α in serological specificity. Therefore, from this, it is tentatively concluded that *M. lepraemurium* belongs to the Avian group in the present serological typing of mycobacteria.

In connection with this tentative conclusion, the report of ASAMI et al. (1959) is of interest. These workers noted the similiarity of *M. lepraemurium* and *M. avium* in terms of the skin reaction patterns revealed by cross testing with their antigens. However, the question of whether or not *Lepraemurium* α is entirely identical with *Avian* α can only be decided by further immunochemical analyses of *Avian* α with anti *Lepraemurium* α serum.

The location of *Lepraemurium* α in murine leprosy cells is unknown. However, since α antigen of *M. tuberculosis*, originally isolated from the culture filtrates, has been shown to be a surface component of the organism (FUKUI *et al.*, 1965), *Lepraemurium* α may also be present on/in the cells of *M. lepraemurium* as releasable surface component. Rees et al. (1965) reported the demonstration of production of the soluble antigens by *M. lepraemurium* using tissue culture methods, in which they could detect some antigenic components in common with those of *M. tuberculosis*. It will therefore be of particular interest to examine the soluble antigens released by the organism into the tissue culture fluid and to see if they contain *Lepraemurium* α.

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FIGURE 1 a Immunodiffusion pattern demonstrating the presence in *M. lepraemurium* of the *crm* whose antigenic determinants are partially in common with α.

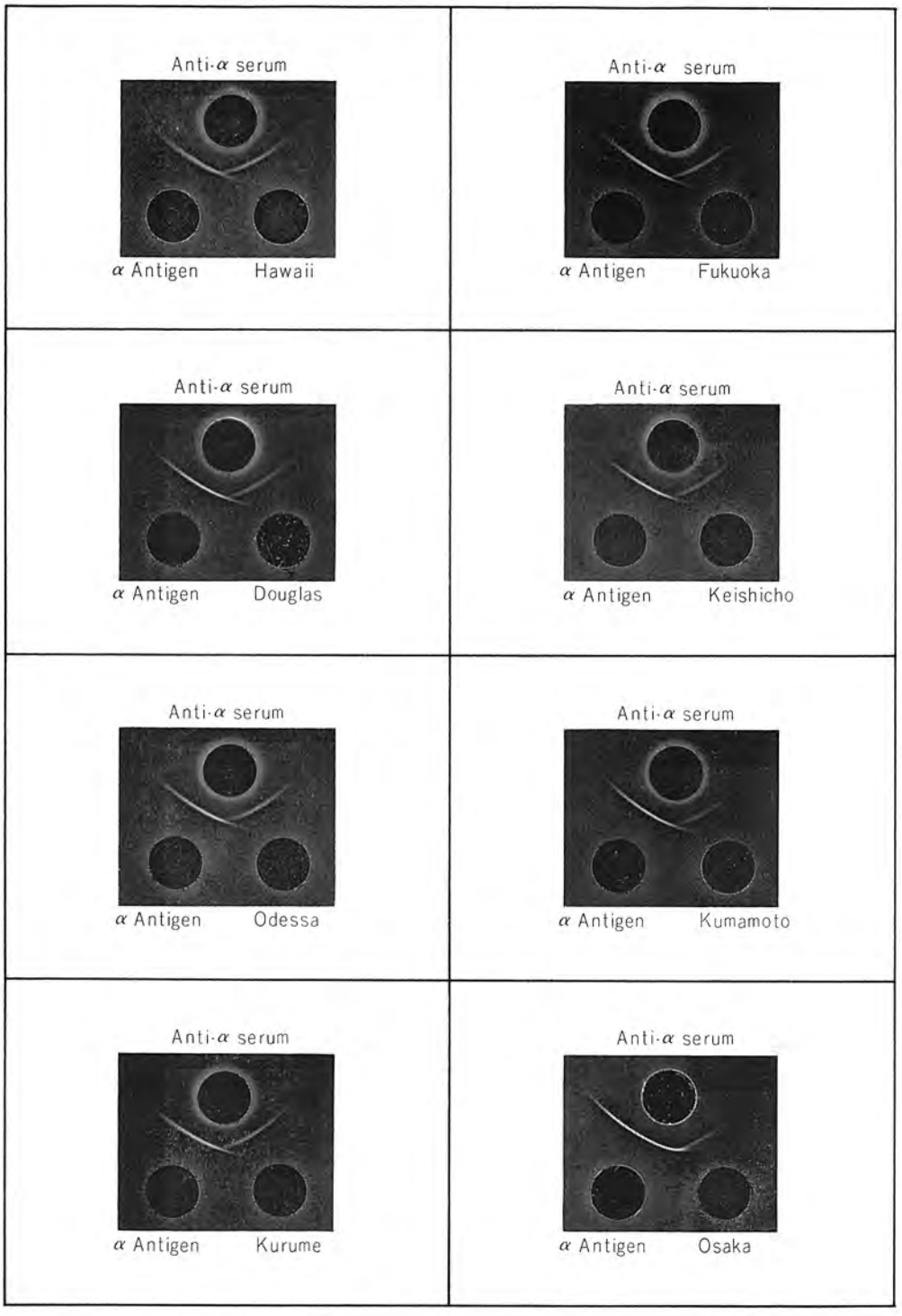

Anti-α serum
α Antigen
Hawaii
Anti-α serum
α Antigen
Fukuoka
Anti-α serum
α Antigen
Douglas
Anti-α serum
α Antigen
Keishicho
Anti-α serum
α Antigen
Odessa
Anti-α serum
α Antigen
Kumamoto
Anti-α serum
α Antigen
Kurume
Anti-α serum
α Antigen
Osaka

FIGURE 1 b Immunodiffusion pattern demonstrating the absence of β antigen in *M. lepraemurium*.

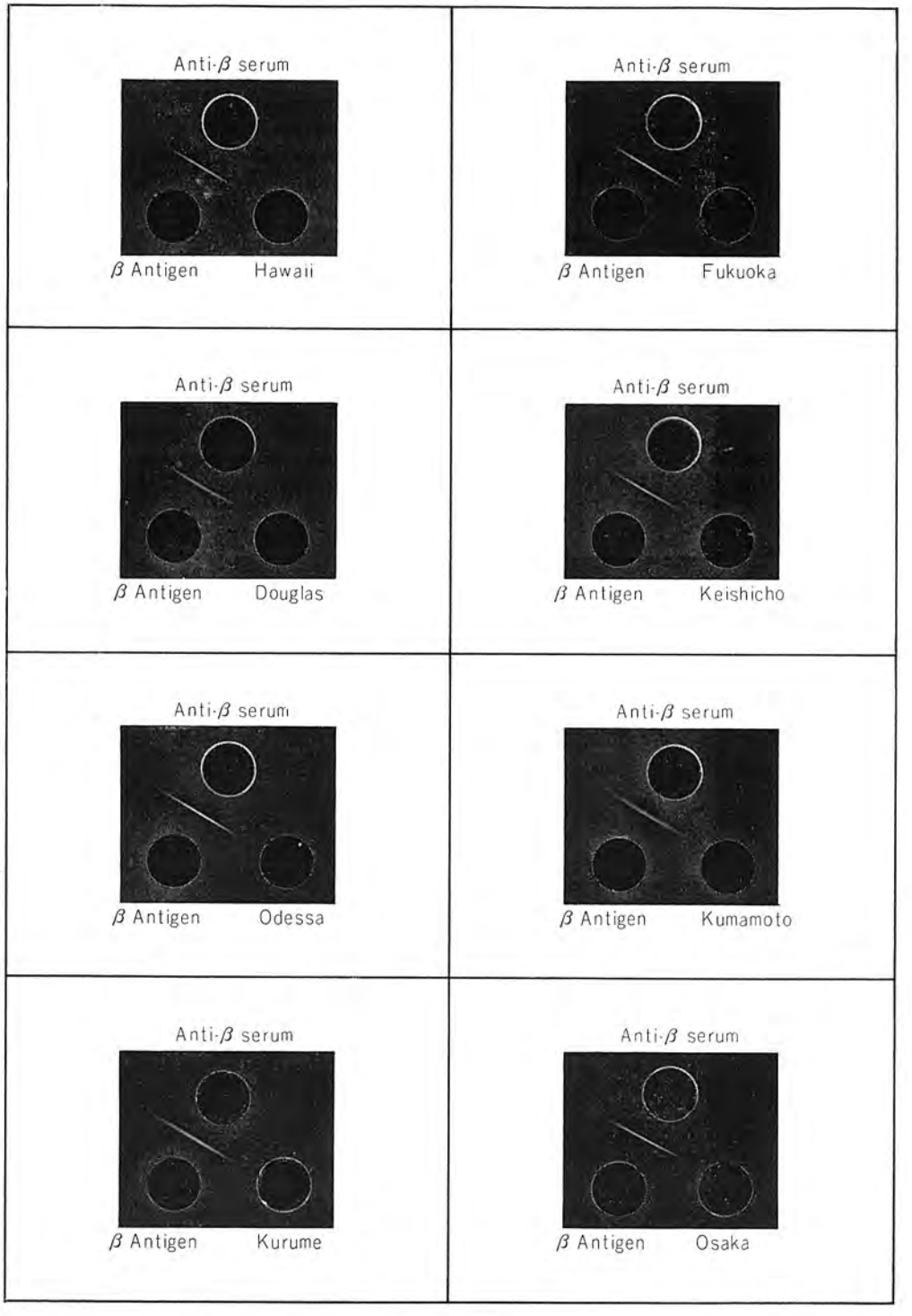

Anti-β serum
β Antigen Hawaii
Anti-β serum
β Antigen Fukuoka
Anti-β serum
β Antigen Douglas
Anti-β serum
β Antigen Keishicho
Anti-β serum
β Antigen Odessa
Anti-β serum
β Antigen Kumamoto
Anti-β serum
β Antigen Kurume
Anti-β serum
β Antigen Osaka

FIGURE 2 Immunodiffusion pattern demonstrating the antigenic difference between P8 α and *Lepraemurium* α.

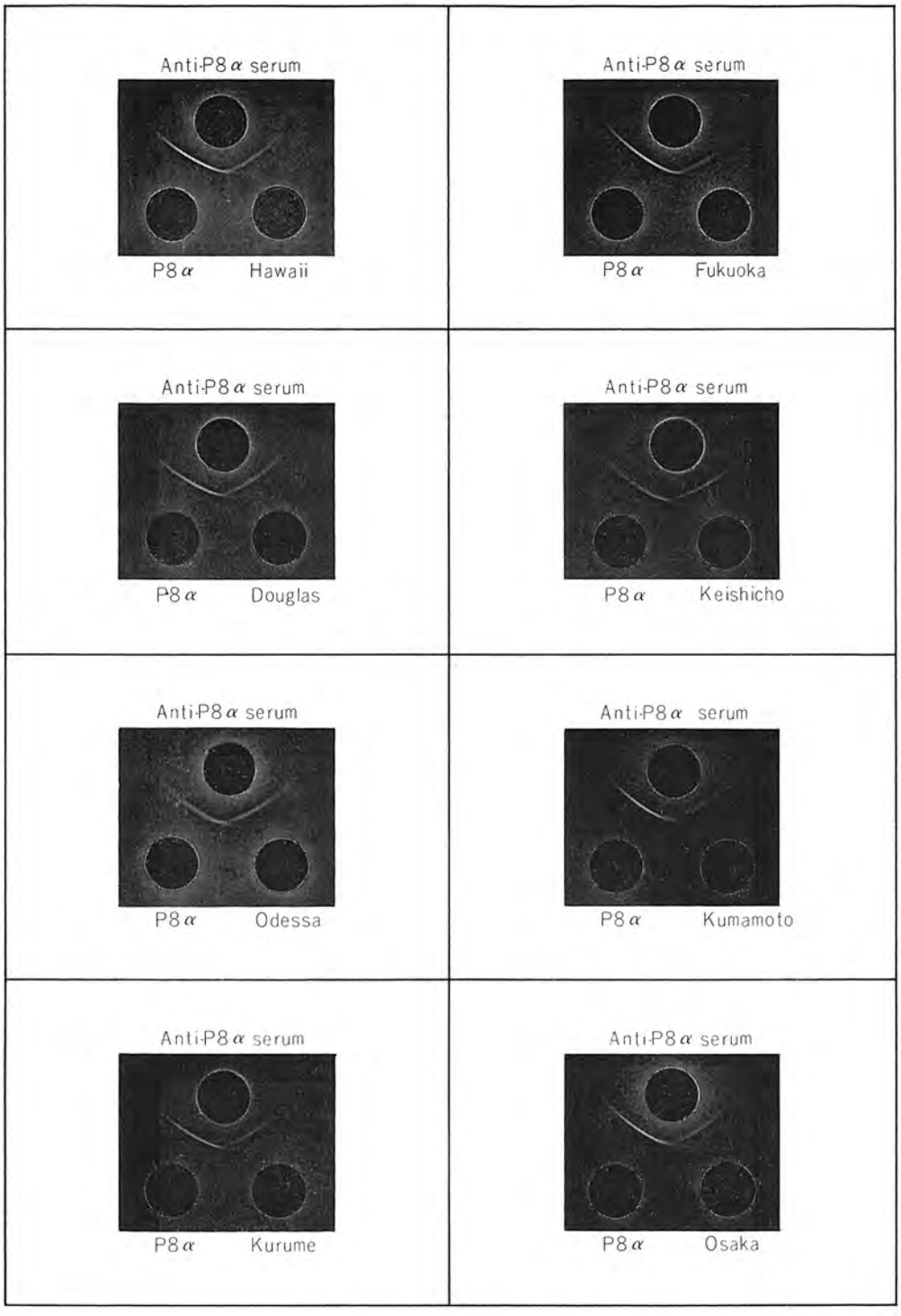
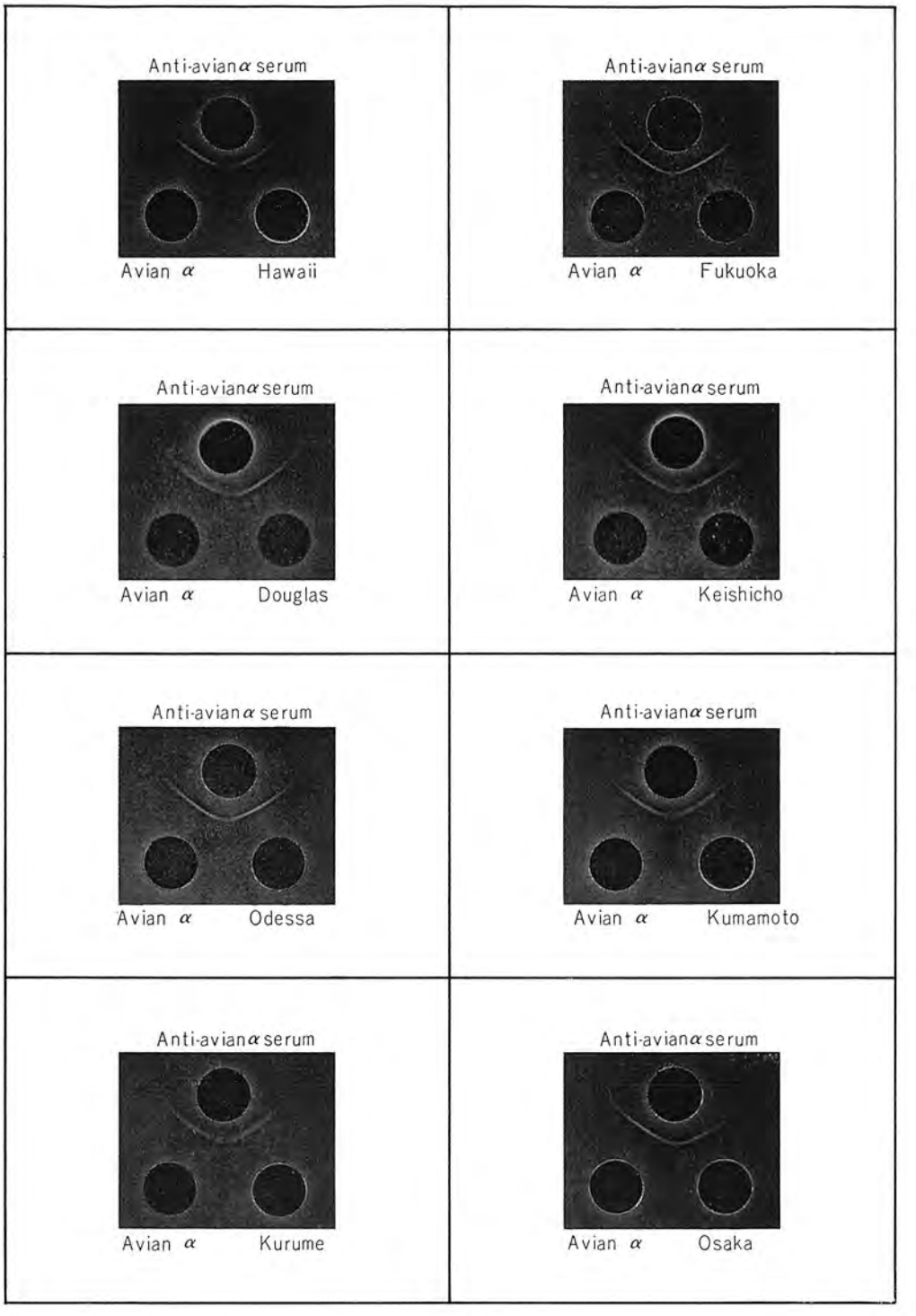

Anti-P8α serum
P8α Hawaii
Anti-P8α serum
P8α Fukuoka
Anti-P8α serum
P8α Douglas
Anti-P8α serum
P8α Keishicho
Anti-P8α serum
P8α Odessa
Anti-P8α serum
P8α Kumamoto
Anti-P8α serum
P8α Kurume
Anti-P8α serum
P8α Osaka

FIGURE 3 Immunodiffusion pattern demonstrating the identity of *Lepraemurium* α with Avian α.


Anti-avian α serum
Avian α Hawaii
Anti-avian α serum
Avian α Fukuoka
Anti-avian α serum
Avian α Douglas
Anti-avian α serum
Avian α Keishicho
Anti-avian α serum
Avian α Odessa
Anti-avian α serum
Avian α Kumamoto
Anti-avian α serum
Avian α Kurume
Anti-avian α serum
Avian α Osaka