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Biological Properties of a *Chlamydia* Strain Isolated from a Pet Bird, Budgerigar Which Was Kept by a Psittacosis Patient

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ABSTRACT. A newly isolated *Chlamydia*, designated Izawa strain, was compared with the meningopneumonitis (MP) strain in morphology, serology and biophysical properties. The fine structure of the Izawa strain differed somewhat from the MP strain in the number of surface projections on the surface of elementary bodies (EBs), whereas no obvious difference was seen in the morphology of the chlamydia cells and intracytoplasmic inclusions. When EBs were centrifuged in linear gradients, the Izawa-EBs formed a band at a density of 1.19 g/ml, whereas the MP-EBs banded at a density of 1.21 g/ml. Reticulate bodies (RBs) of the Izawa strain were much more rigid to ultrasonic treatment which completely disintegrated the MP-RBs. There was no difference in the antigenicity between both strains when anti-Izawa rabbit or human serum was used, indicating the usefulness of the MP strain as antigen for the epidemiological survey and serodiagnosis of human sera.

It is well known that *Chlamydia psittaci*, one of the pathogens of zoonoses, causes psittacosis and related diseases in man. The organisms, obligate intracellular prokaryotic parasites, multiply through a unique, complex growth cycle in which there are two different cell types, one is infectious elementary body (EB) and the other is vegetative, reticulate body (RB).[1] The EB penetrates a susceptible animal cell and is converted into a large RB which multiplies by binary fission, then undergoes maturation *via* an intermediate body to form EB. Both cell types differ from each other not only in morphology, but also in many respects, including density, strength against mechanical agitation, cell wall permeability and chemical composition in the cell envelopes.

Through careful investigations of newly isolated *Chlamydia* organisms from a pet bird, a budgerigar which was kept by a patient suffering from severe psittacosis, we found that the organism was a member of *C. psittaci* and that some biological properties of the organisms differed from those of *C. psittaci* meningopneumonitis (MP) strain which was well investigated. A comparative study on the morphology and the other biological properties of the two strains is reported in this paper.

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MATERIALS AND METHODS

Organisms. A new strain, which was designated Izawa-1 strain and used throughout this study, was one of the isolates from three budgerigars which were kept by a psittacosis patient. The clinical figures of the patient will be reported in another paper.

The spleen showing severe splenomegaly (about 8 mm in diameter) was taken out, homogenized in a glass homogenizer, and Eagle's minimum essential medium (MEM, Nissui Chemical Co., Tokyo, Japan) containing 2% heat inactivated fetal calf serum (FCS, GIBCO Laboratories, USA) was added to make 10% homogenate, followed by centrifugation at $600 \times g$ for 10 min to remove large cell debris. The supernatant (1 ml) was overlaid on a monolayered L cell culture in a plastic culture dish (35×10 mm in size), which was incubated at 37°C in a CO_2 (5%)-incubator. After 3 days, the culture medium was changed with fresh MEM containing 2% FCS. Careful examinations revealed that the multiplication of the new strain was strongly affected with the FCS used, indicating the necessity to closely select the commercially prepared FCS for isolation of wild chlamydia strain. The MP strain was cultivated in the L cell cultures as previously described.[2] Tawma strain of *C. trachomatis*, which was isolated from a trachoma patient in Burma by Higashi[3] and maintained by yolk sac inoculations, was also used.

Purification of EBs. Suspension L cells maintained in our laboratory for about 20 years were highly susceptible to both the Izawa and MP strains. The EBs of these strains were, therefore, purified from the suspension L cell cultures harvested at 48 hours for the MP and at 72 hours for the Izawa strain postinoculation by the method of Tamura and Higashi.[2]

Inoculation of embryonated eggs. To examine the sensitivity of the Izawa strain against sulfadiazine, 0.5 ml of suspension [0.9 IFU (inclusion forming units)/cell] of the Izawa strain was injected into yolk sacs of 6 day-old eggs. Soon after injection, 1 mg of sulfadiazine (kindly supplied by Daiichi Seiyaku Co. LTD., Tokyo, Japan) in 1 ml of MEM (pH 9.5 adjusted by the addition of NaOH to facilitate solubility of the drug) was injected into the eggs and then incubated at 37°C. As the positive control, the Tawma strain (This strain has not been propagated in any cell cultures. Therefore, the inoculum of this strain was prepared from an infected yolk sac harvested on 7th to 10th day postinoculation. Ten percent homogenate in MEM without FCS was prepared and 0.5 ml of the homogenate was used as the inoculum, which infectivity was about 5×10^4 LD_{50}/ml. This homogenate was diluted appropriately before use.) was also injected into yolk sacs, and the injected eggs were treated identically with those injected with the Izawa strain. When the eggs died, Giemsa and Macchiavello staining methods were used to detect the chlamydial cells in yolk sac smears.

Light microscopy. To observe the intracytoplasmic inclusions, monolayer culture of L 929 cells or HeLa 229 cells on a cover slip in a Leighton tube was inoculated. At intervals of postinoculation, the cells were stained by Giemsa staining and iodine staining procedures. In some observations, the centrifugation at $1,500 \times g$ for 30 min was done for inoculation to the HeLa cells by the method previously reported.[4]

Immunofluorescent antibody technique. Three antisera were used. One was obtained from the psittacosis patient who kept the birds used for the isolation of the new strain. The other antisera against the Izawa and MP strains were prepared from male New Zealand rabbits that had received 4 weekly intramuscular injections of the purified EBs (total protein of about 3 mg/rabbit, for each strain) in Freund complete adjuvant. To examine the antibody titer, a newly established immunofluorescent antibody titration method, namely microplate immunofluorescence antibody (MFA) technique,[5] was carried out. Briefly, cells in a glass culture bottle were incubated at 37°C after being infected with an inoculum that produced inclusions in 90% and more of the cells. At 26 hours postinoculation, the cells were dispersed with a trypsin-EDTA solution, seeded into wells in a Terasaki plate (Flow Laboratories Inc., USA) and then allowed to form monolayer in the CO_2-incubator for 2 hours. After ethanol fixation for 30 min, each well was overlaid with a serially diluted antiserum solution for 1 hour in a moist chamber. After several washings with PBS, the cells were stained for 1 hour with commercially prepared FITC-labeled sheep antihuman or antirabbit globulin (Medical and Biological Laboratories, LTD., Nagoya, Japan). After having been washed with PBS, the plate was mounted in glycerol and examined by dark field fluorescent microscopy. The antiserum titer was readily determined by the dilution endpoint for highly specific staining of inclusions.

Electron microscopy. The infected cell cultures in suspension harvested at intervals were sedimented with low-speed centrifugation ($300 \times g$) for 5 min. The cells in a pellet were doubly fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated in an ethanol series and then embedded in Spurr's epoxy resin.[6] Thin sections were cut on a Porter-Blum microtome and stained with uranyl acetate in 50% ethanol and lead citrate solution. The purified EBs collected into a pellet were treated by the method above mentioned. When the projections on the EB surface was examined in thin sections, the purified EBs in a pellet were treated with tannic acid by the method reported previously.[7] For freeze-replica preparations, the infected cells in pellets were fixed in the glutaraldehyde fixative, impregnated with 10% glycerol, frozen in Freon 22 at liquid nitrogen temperature and then transferred into liquid nitrogen. The replica was prepared in a Balzers BAF 301 freeze-replica apparatus by the method reported previously.[8] When the fractions obtained by the sucrose density gradient centrifugation were examined, the samples were mounted on a grid by the agar method described by Sharp[9] and shadowcast with Pt-palladium alloy in a JEE 4X (JEOL Lit., Tokyo, Japan) high vacuum evaporator. The thin sections were also prepared by the identical procedures with those for the infected cells and purified EBs. All specimens were examined under a Hitachi H-500 transmission electron microscope at 75 kV.

To determine the number of projections on the EB surface, the purified EBs of the Izawa strain were mounted on a nickel grid which was treated with poly-L-lysine (0.01% in distilled water), and fixed doubly with 1.8% glutaraldehyde and 2.5% osmium tetroxide. EBs on the grid were dehydrated in an ethanol series, dried with CO_2 in a Hitachi HCP-1 critical point dryer, followed by the coating of Au-palladium alloy on a rotating stage in a Hitachi-4GB high vacuum evaporator. The specimen was examined with a Hitachi S-570

scanning electron microscope at 25 kV. EBs in micrographs in which one could view the whole surface area containing projections were selected, and the number of projections was counted for statistical analysis.

RESULTS

Morphology of Izawa strain. Intracytoplasmic inclusions formed in the L cells and HeLa cells were identical not only in morphology, but also in stainability with the MP inclusions (Fig. 1). The cytoplasm of infected cells was occupied with large inclusions which were well stained with Giemsa staining method,

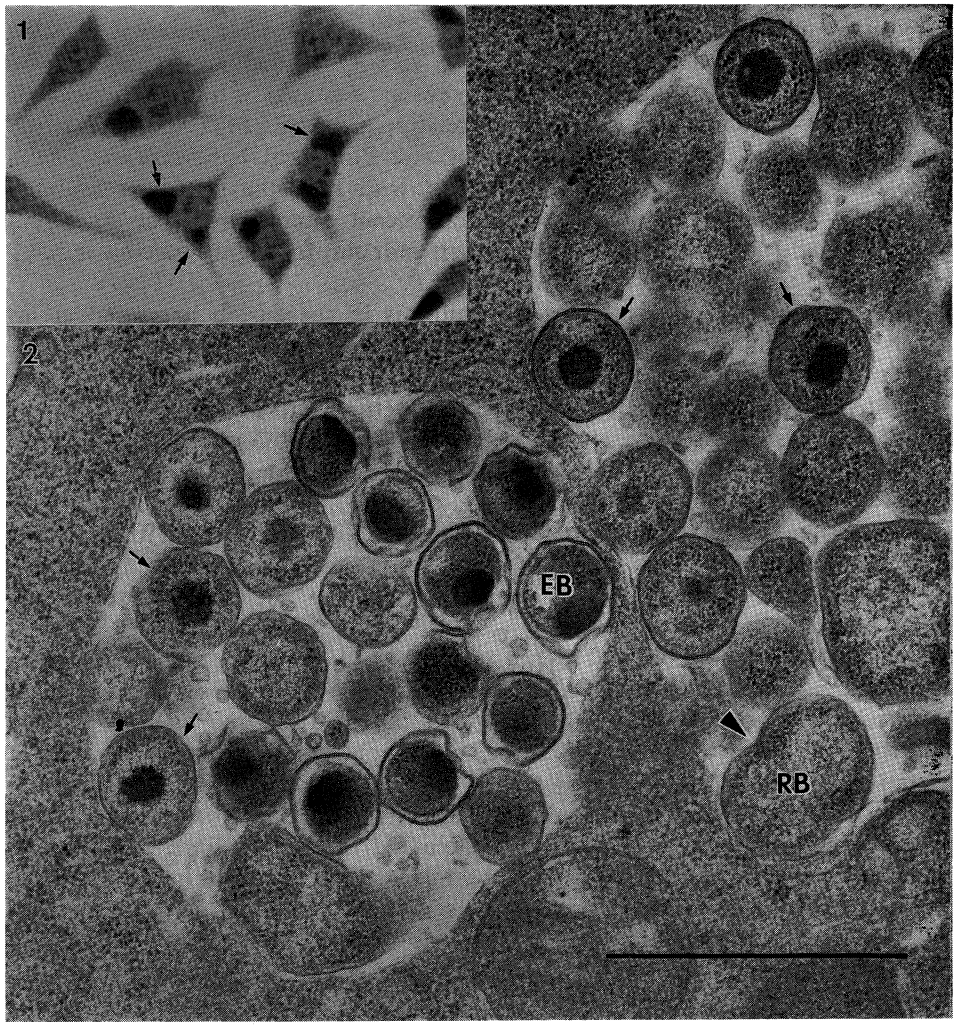

Fig. 1. L cells fixed with methanol at 24 hr postinoculation and stained by Giemsa method. Inclusions (arrows) are clearly seen in the cytoplasm.
Fig. 2. Inclusions in L cell harvested at 40 hr postinoculation. The inclusions contain EBs, RBs and intermediate bodies (arrows). Arrowhead indicates RB undergoing binary fission. Bar indicates 1 μm.

whereas no inclusions were stained with iodine staining procedure. The results indicate two important facts for identification of *C. psittaci* ; formation of the unique intracytoplasmic inclusions and lack of glycogen accumulation in the inclusions. Growth rate of the inclusions appeared to be comparable to that of the MP strain. However, distinct cytopathic effect (CPE) after inoculation with the Izawa strain (infected at 0.5 to 1 IFU/cell) occurred at 70 hours and more in the L cell and HeLa cell cultures, whereas CPE with the MP strain was seen at about 40 hours. Fig. 2 shows inclusions in a L cell harvested at 40 hours postinoculation. There are three cell types. The smaller dense forms are EBs (0.3–0.35 μm in diameter) containing fine particles (ribosomes) and a dense nucleus which is located at the eccentric region. The second form is RB less dense and large in size (0.5–1 μm in diameter). One of them appears to be undergoing binary fission. The third form of cell types is intermediate body which is in maturation phase to form EB from RB. The intermediate bodies are characterized by the presence of dense nucleus at the center of cell. These morphology, together with the presence of inclusion membrane, well agreed with that of the MP strain. Fig. 3 shows purified Izawa EBs at higher magnification. Their morphology was identical with that of the purified MP-EBs in many respects, such as location of nucleus, thickness of cell wall, electron density of nucleus and cytoplasm, and dimension. When the EBs were treated with tannic acid and examined in the thin section, many surface projections were clearly seen (Fig. 4). No difference was recognized between the Izawa and MP strains in dimension nor in the arrangement of the projections on the

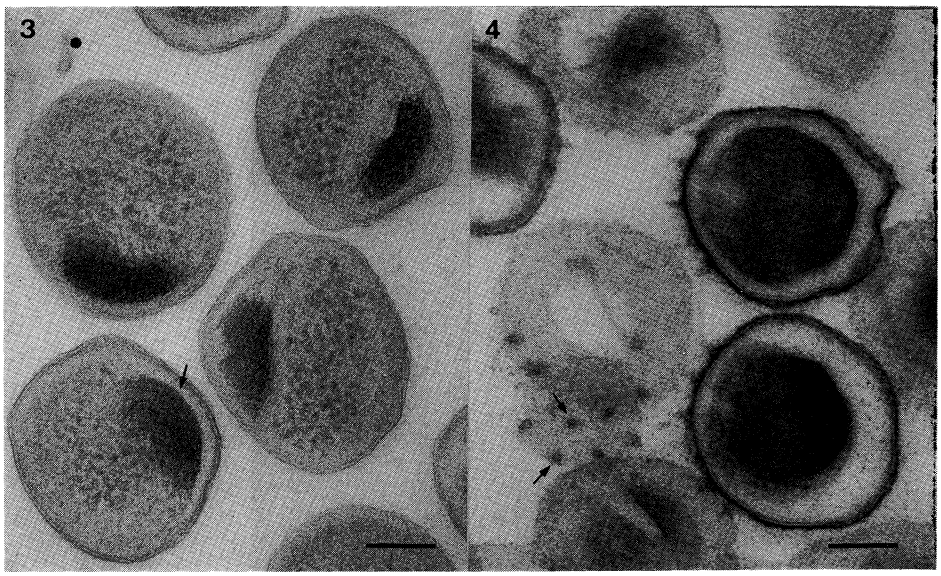

Fig. 3. Purified EBs at higher magnification. The EBs were fixed doubly and stained doubly. The nucleus (arrow) is located in an eccentric region of cytoplasm. Fine particles are ribosomes. No projection is seen on the surface of EBs. Bar indicates 100 nm.

Fig. 4. EBs treated with tannic acid. Many projections are visible on the surface of EBs, whereas internal structure in each EB is not clearly distinguished for the high density resulted from tannic acid treatment. Some projections are cut crossly (arrows). Bar indicates 100 nm.

EB surface. It is, however, hard to determine the number of projections in thin sections. Therefore, scanning electron microscopy was carried out. Fig. 5 shows one of the micrographs which were taken for counting the number of projections. The projections were arranged hexagonally with a center-to-center spacing of 40 to 70 nm. The distribution of the number of projections per MP-EB and per Izawa-EB is shown in Fig. 6. On the Izawa-EBs, there was a peak at 22 to 24 projections, and the average number of projections was 22.1, whereas the average number of the MP-EBs was 17.5, and a peak was seen at 16 to 18 projections.[10]

The freeze-replica of infected cells was prepared at 30 hours postinoculation. Fig. 7 shows an inclusion containing EBs and RBs of the Izawa strain. All convex surfaces were covered with fine particles, whereas no particles were seen on the concave surfaces as was reported previously.[11] The B structures, which have been determined as the morphological markers of EB,[11] were visible only on some concave surfaces. It is noted in the figure that the B structures in two groups are seen on one of the concavities. Such an arrangement was never encountered in EBs of the MP strain, but we could find a similar arrangement of the B structures in EBs of the *C. trachomatis* TE-55 strain.[12] Matsumoto and Higashi[13] reported that the MP-EBs possessed the projections in a limited surface area. The scanning electron microscopy in the present study showed that the Izawa-EBs normally possessed the projections

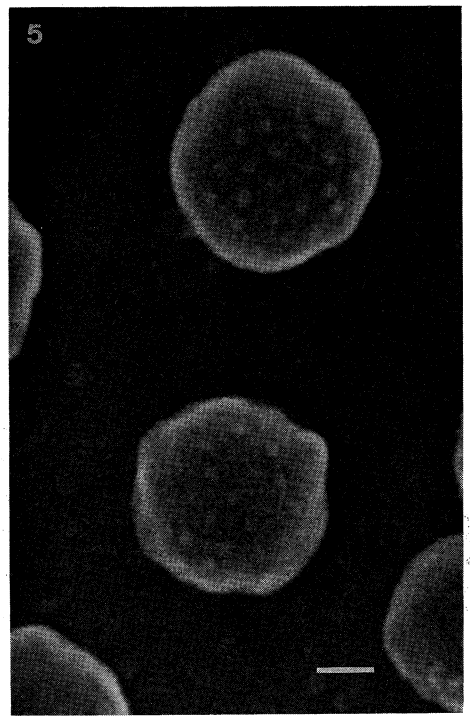

Fig. 5. EBs observed by scanning electron microscopy. Projections are seen in a group, in which the projections arrayed hexagonally with a center-to-center spacing of about 40 to 70 nm. Bar indicates 100 nm.

within one surface area. Therefore, the EB having two groups of the projections seemed to be an aberrant form. The cleavage of frozen specimen was occasionally occurred in the inclusion membrane as shown in Fig. 8, in which a convex surface of an inclusion is exposed. Many fine particles in groups are seen.

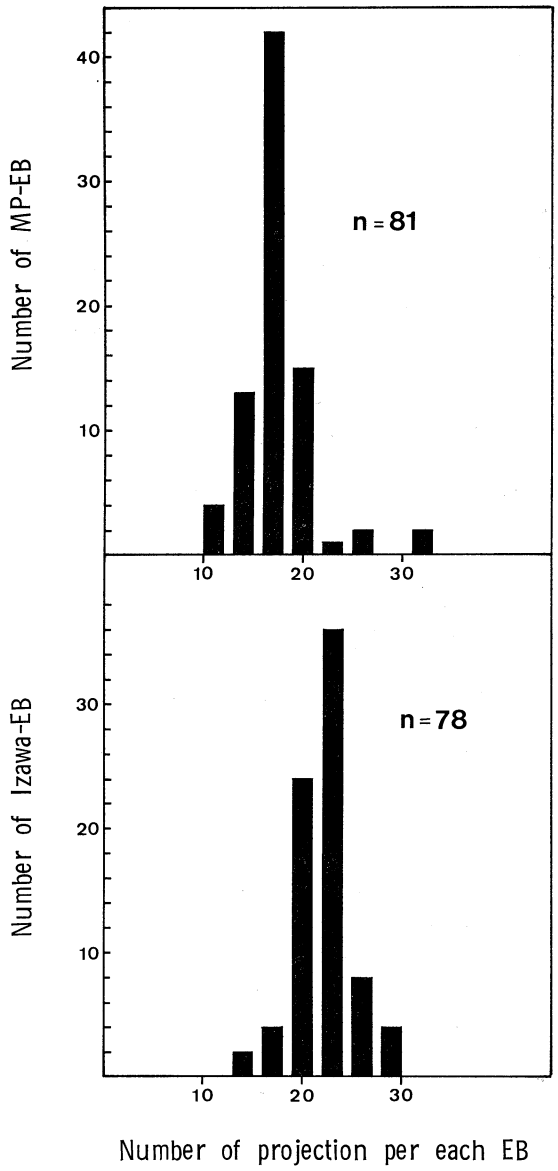

Fig. 6. Distribution of the number of projections per EB of Izawa and MP strains. The histogram of the MP-EB is cited from the results previously reported.[10] Each bar indicates the total number of EB having 10 to 12 and 13 to 15 projections, etc. It is clear that the average number per Izawa-EB and the range of the projection number are different from those of the MP-EB.

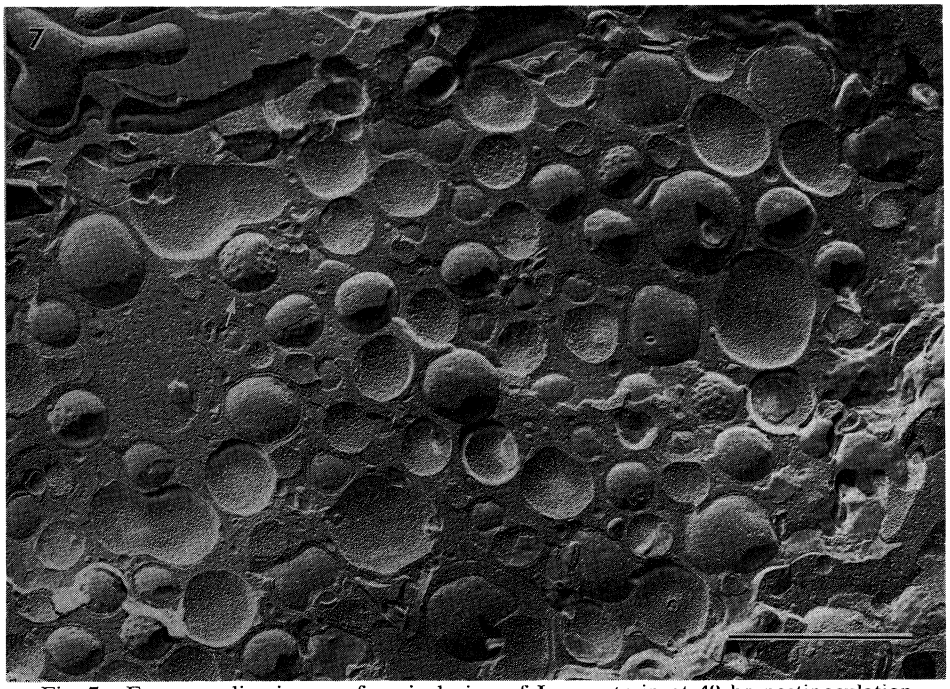

Fig. 7. Freeze-replica image of an inclusion of Izawa strain at 40 hr postinoculation. Many convex and concave surfaces are seen. On some concave surface, the B structures in a hexagonal arrangement are visible. Arrow indicates a concavity having two groups of the B structures. Bar indicates 1 μm.

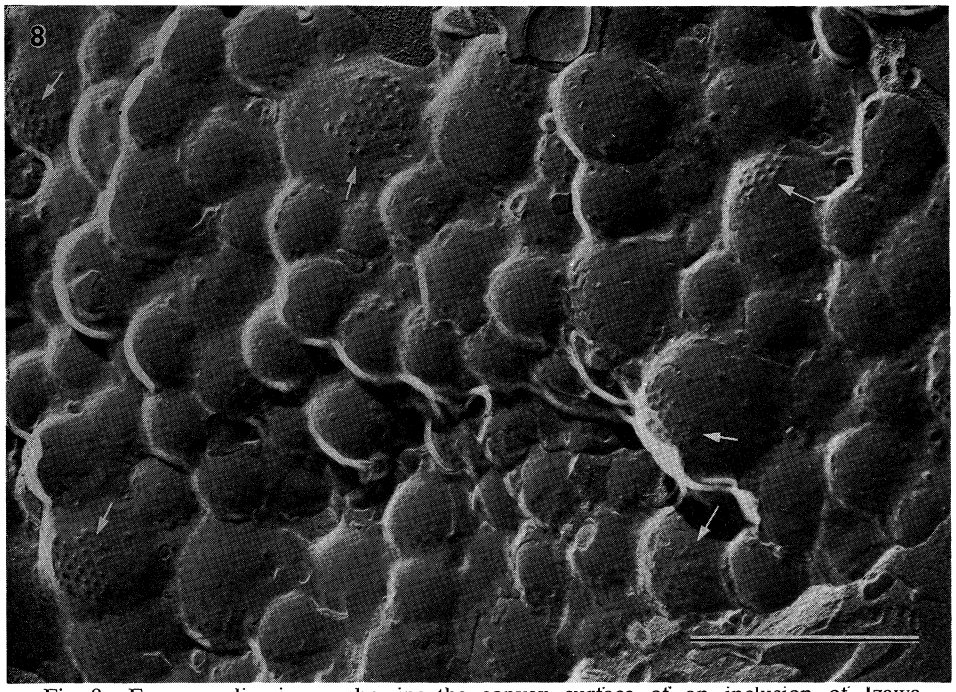

Fig. 8. Freeze-replica image showing the convex surface of an inclusion of Izawa strain. Particles in groups are indicated with arrows. The surface shows ruggedness along RB outlines. Bar indicates 1 μm.

These particles have been identified as the RB projections which pierced the inclusion membrane.[8]

Sensitivity to sulfadiazine. As shown in Fig. 9, the survival of eggs in the presence of sulfadiazine was exactly the same with that of eggs in the control group, whereas the Tawma strain showed the prolongation of survival in the presence of the drug. The multiplication of the chlamydiae was confirmed in all dead eggs by the staining methods. The results indicated that the Izawa strain was not sensitive to sulfadiazine. This fact demonstrates one of the criteria for identification of *C. psittaci.*

Antigenic property of Izawa strain. The L cell monolayers at intervals after inoculation were stained by indirect immunofluorescent antibody techniques. As shown in Fig. 10, the inclusions of the Izawa strain were specifically stained not only with anti-Izawa-EB rabbit serum, but also with anti-MP-EB rabbit serum and the serum collected from the psittacosis patient. These results strongly suggested that the Izawa strain possessed common antigen(s) with the MP strain and that the patient was suffering from the Izawa strain. Table 1 shows the

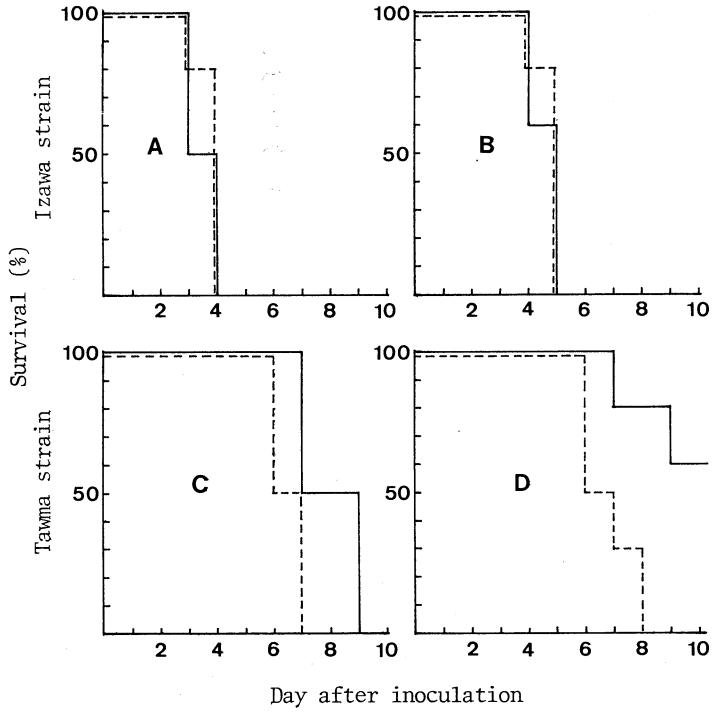

Fig. 9. Effect of sulfadiazine on the survival of embryonated chicken eggs inoculated with Izawa and Tawma strains. Ten eggs were used in each experiment. Five (experimental group) of them were injected with the inoculum (A : 1.5×10^4 IFU/egg, B : 1.5×10^3 IFU/egg, C : 2.5×10^3 LD_{50}/egg, D : 2.5×10^2 LD_{50}/egg) and sulfadiazine (1 mg/ml of MEM, each) (solid line). The other eggs (control group) were also injected with the inoculum and 1 ml of MEM instead of the drug solution (broken line). No difference in survival between the experimental and control groups was seen for Izawa strain, whereas the survival of eggs inoculated with Tawma strain was prolonged by the injection of the drug, depending on the dose of inoculum.

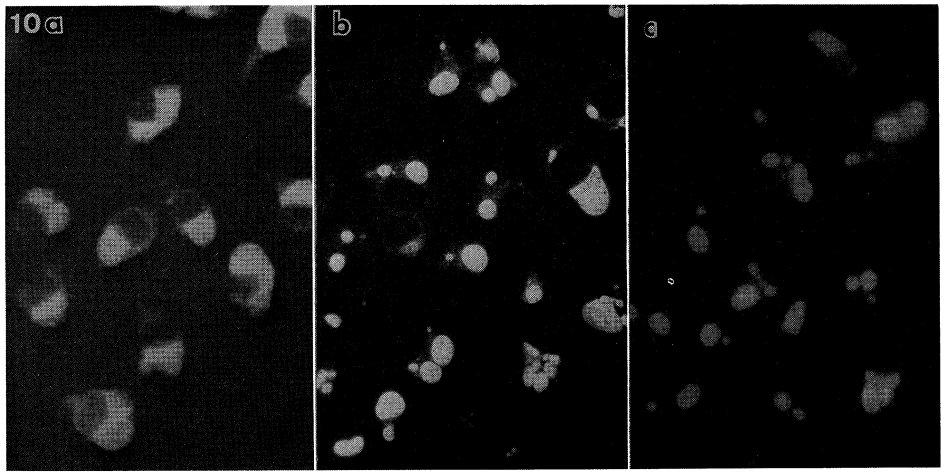

Fig. 10. Inclusions of Izawa strain stained with anti-Izawa rabbit serum (a), anti-MP rabbit serum (b), and serum collected from the psittacosis patient (c).

TABLE 1. Antibody titers of anti MP and anti Izawa sera assayed with different strains by MFA technique

Antisera		Antigens	
		MP-inclusions	Izawa-inclusions
anti MP rabbit serum	No. 1*	16,384	512
	No. 2	4,096	1,024
	No. 3	16,384	8,192
anti Izawa rabbit serum	No. 1	4,096	4,096
	No. 2	16,384	16,384
	No. 3	16,384	16,384
patient sera collected on	Dec. 13′ 82	2,048	2,048
	Dec. 15′ 82	2,048	2,048
	Dec. 24′ 82	4,096	4,096

*Each number indicates the serum obtained from an immunized rabbit.

antibody titers assayed by the MFA technique. When anti-Izawa sera were assayed with the MP and Izawa strain as antigens, no difference was detected in titers between either strain. Similarly, no difference was detected in the two strains with the patient sera. However, when anti-MP rabbit sera were assayed with the Izawa strain, the titers were regularly lower than those assayed with the MP strain. From these results, it seemed that the Izawa strain possessed common antigen(s) with the MP strain and that the MP strain was capable of being used as a safe antigen for the titration of patient sera by the MFA technique.

Biophysical properties of Izawa strain. As shown in Fig. 3, EBs of the Izawa strain were recovered in high purity from the infected L cell suspension cultures by the purification procedures for the MP-EBs. After inoculation with the Izawa strain, the cells were harvested at 48 hours and 72 hours, sonicated (20 kc/sec) in a Kubota BH-200P sonicator for 30 seconds and then spun down

at 1,000×g to remove cell debris. The supernatant was overlaid on a 25% sucrose cushion and centrifuged at 10,000×g for 60 min. The pellet was suspended in 0.2 M Tris-HCl buffer pH 7.3 containing $MgCl_2$ (20 mM) and treated with DNase, RNase, and then trypsin at 37°C, followed by linear sucrose density gradient (20-50%, w/v) centrifugation in a RPS-65T rotor at 10,000 rpm for 90 min. Two bands were formed in each column (Fig. 11-a). The diffuse top band contained a number of EBs in high purity and the sticky bottom band was formed mainly with RBs and cell debris containing some EBs. It was noted that the bottom band of 72 hour-sample was slightly lower than that of 42 hour-sample. When the bottom bands were treated with 0.5% sodium dodecyl sulfate (SDS), a number of RB and EB cell walls were recovered. When the cells infected with the MP strain were similarly treated, a clear EB band was obtained in the gradient columns, but no bottom band was obtained. These result indicated that the Izawa-RBs were stable against sonication which completely disintegrated the MP-RBs. To remove the bottom band, sonication for 90 seconds and more was needed. To compare the density, the purified EBs of both strains were put on the sucrose columns and centrifuged at 10,000 rpm in the RPS-65T rotor for 120 min. The result is shown in Fig. 11-b. A band of the Izawa-EBs was formed at a site slightly higher than that of the MP-EB band, indicating that the density of the Izawa-EB was slightly lower than that of the MP-EB. This is more clearly shown in Fig. 12, where the fractions collected from the tube bottom were assayed by spectrophotometry at 260 nm of wave length. The Izawa-EBs banded in the gradient at a density of 1.19 g/ml, whereas the MP-EB banded at a density of 1.21 g/ml. The value of the MP-EB density agreed with that reported by Fris.[14] It is, therefore, concluded that the density of the Izawa-EB is slightly lower than that of the MP-EB.

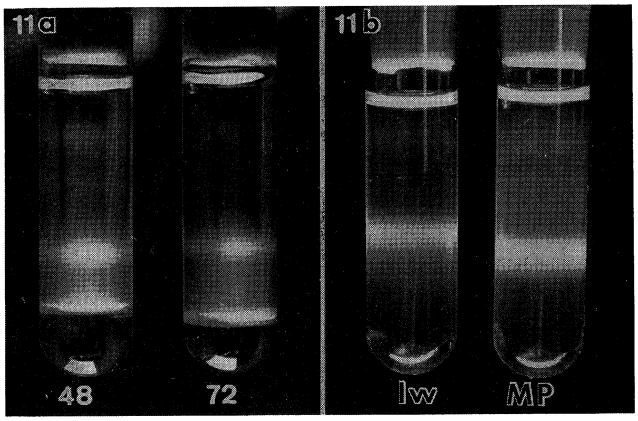

Fig. 11. Sucrose gradients showing bands after centrifugation of Izawa and MP strains. (a) Top bands contained EBs in high purity. Bottom bands in both samples harvested at 48 and 72 hr appeared to be sticky and was formed with a number of RBs and cell debris. The bottom band of the 72 hr-sample is slightly lower than that of the 42 hr-sample. (b) The band of Izawa EBs (Iw) is slightly higher than that of MP-EBs (MP), indicating that the density of Izawa EB is lower than that of MP-EB.

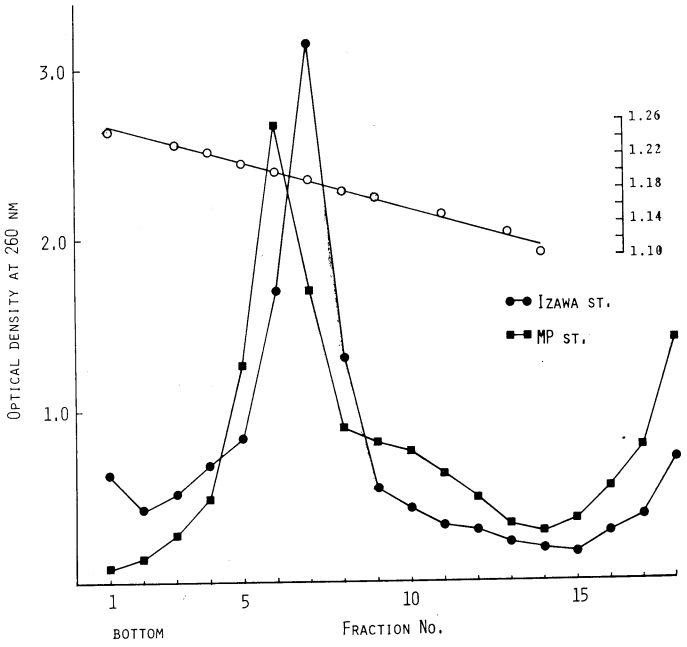

Fig. 12. Fractionation of Izawa EBs (●—●) and MP-EBs (■—■) by sucrose density gradient centrifugation. The Izawa-EBs banded at a density of 1.19 g/ml, whereas the MP-EBs banded at a density of 1.21 g/ml.

DISCUSSION

The biological properties of the Izawa strain were summarized as follows : (i) The morphology and stainability of the intracytoplasmic inclusions were identical with those of MP strain. Although the growth rate of the inclusions of both strains appeared to be very similar to each other, the Izawa strain needed about one day longer than the MP strain to cause evident CPE in the L cell monolayer cultures. (ii) The fine structure of cell types of the new strain was also identical with that of the MP strain, except in the number of surface projections. (iii) The density of the Izawa-EB in sucrose solution was slightly lower (1.19 g/ml) than that of the MP-EB (1.21 g/ml). (iv) The Izawa-RBs were much more rigid than the MP-RBs to the mechanical agitation, and (v) the Izawa strain showed antigenicity identical with the MP strain when anti-Izawa rabbit or human serum was used. However, the antibody titers assayed by combination of the Izawa strain and anti-MP rabbit sera were regularly lower than those obtained by the homologous combination of the MP strain and anti-MP sera.

From these facts, together with the stable multiplication in the presence of sulfadiazine and the lack of glycogen accumulation in the inclusions, it was concluded that the Izawa strain was a member of *C. psittaci* strains. Based on the high titer and specificity of the patient serum assayed by the immunofluorescent antibody staining, it was strongly suggested that the Izawa strain was the

agent which caused psittacosis in the patient. The results obtained in the serological experiment indicated the usefulness of the MP strain as a readily preparable, safe antigen for the epidemiological survey and serodiagnosis of human sera.

The only difference detected in morphology between the Izawa and MP strains was in the number of projections on the EB surface. Matsumoto reported that each MP-EB possessed 17.5 projections in the average number[13] and that the projections were bound to DNA molecules.[15] Gregory *et al.* reported the presence of the surface projections on EBs of two strains of *C. psittaci* and four strains of *C. trachomatis*, and described the usefulness of the projections as the phenotypic marker for recognizing members of genus *Chlamydia*.[16] Soloff *et al.* confirmed the presence of the projections in the *in situ* organisms of guinea-pig inclusion conjunctivitis (GPIC) agent, a *C. psittaci* strain.[17] The MP-RBs also possess the surface projections which pierce the inclusion membrane and connect directly with the host cytoplasm.[10] The biological meaning of the difference in the number of projections between the MP and Izawa strains is obscure. However, the facts mentioned above suggest that the projections play an important role during infection and multiplication of chlamydiae.

Acknowledgment

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