IMMUNOLOGICAL STUDIES OF ISOLATED PARTICULATES OF *PARAMECIUM AURELIA*

I. Antigenic Relationships Between Cytoplasmic Organelles and Evidence for Mitochondrial Variations as Demonstrated by Gel Diffusion

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ABSTRACT

Mitochondria and other particulates—cilia, trichocysts, and "small granules"—have been isolated from several stocks of *Paramecium aurelia*, syngen 2. Antisera against these particles and against breis have been used to characterize the fractions by diffusion in gel. Evidence is presented for the relationship of particles, as demonstrated by immunologic cross-reactivity of the soluble antigens extracted from them. Although some antigens are unique for a fraction, cross-reacting antigens in two or more fractions, as determined by "spur" formation in agar, suggest a relationship between morphologically diverse particles. A procedure for studying cross-reactions in gels is described using the specific immobilization antigens as a model. The localization of these antigens within cilia, and perhaps trichocysts, has been confirmed. Other organelles, specifically mitochondria and "small granules," appear to alter their specificity spontaneously and reversibly during cell reproduction, a pattern reminiscent of the immobilization serotypes which can transform to one another during clonal growth.

INTRODUCTION

Changes in the composition and distribution of the particulates of a cell can be pieced together by studies of fixed specimens or, with less precision and resolution, by phase contrast observations of living material. However, fluxes not discernible as alterations in morphology may be of considerable importance to cellular function. For sensitivity, flexibility, and ease of manipulation, qualitative variations in intracellular populations of particles were determined by immunochemical methods (*cf.* 1, 2, 3) rather than morphological or biochemical analysis, although others have employed these latter techniques with some success (1, 4).

Fractions were isolated from *Paramecium aurelia*, syngen 2, and were designated cilia, trichocysts, mitochondria, and small granules. Gel diffusion techniques were employed to characterize the soluble antigens associated with the particulates.

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Various observations suggest that the individual particulate fractions may be distinguished antigenically from other fractions, although the particulates also probably possess many antigens in common (*cf.* 2, 3). In addition, often a particle may have an antigen related to, although not identical with, the antigen of a different type of particle.

At least one antigen appears to be restricted in its distribution, the immobilization antigen (which is responsible for the agglutination of cilia in the presence of specific antisera causing immobilization of the affected paramecia). An examination of sera prepared against different fractions revealed its presence only in cilia and trichocysts, in agreement with results obtained by Beale and Kacser (6) and Preer and Preer (7) using other techniques. Similar immobilization antigens when found in different strains are not necessarily identical, and the immunological behavior of these cross-reacting "ciliary" antigens was studied as a possible model for antigens associated with other particulates.

One of the more striking examples of variation of cell constituents during vegetative reproduction has been that of the ciliary antigens. The inheritance of these antigens involves a complex interplay of environment, cytoplasm, and nucleus in the determination of the spectrum, specificity, and transformation of immobilization antigenic types, or serotypes (see Beale (5) for review). These studies have provided models for cellular differentiation, models that have not been broadly applied due partly to lack of evidence for the existence of similar systems, either in *Paramecium* or other protista or metazoa. The experiments described below provide additional evidence for variation against a constant genic background, this time of other more ubiquitous cellular components, mitochondria, and "small granules"; this phenomenon closely parallels, in the respects noted thus far, the behavior of the immobilization serotypes. The variations, the advantages, and limitations of applying immunological techniques to studying subcellular structural relationships is also discussed.

MATERIALS AND METHODS

Stocks and Cultures

The following stocks of *P. aurelia*, syngen 2, were employed: 7 from Pinehurst, N. C., 72 from Pyote, Texas, and 197 from Germany. Two derived lines with genes from several stocks, *d*30-1 and *d*28-1, were also used (see Finger (8) for complete description of these lines).

All stocks and derived lines were kept at either 17° or 27°C., as the experiment required, in a 0.075 per cent Cerophyl (from Cerophyl Laboratories, Kansas City, Kansas) infusion inoculated with *Aerobacter cloacae* (9). Paramecia were grown in 15 liters of culture fluid in jugs and concentrated in a De Laval gyro-tester cream-separator-type centrifuge to a volume of 400 ml. Further concentration was achieved by centrifugation in an International oil testing centrifuge for 2 minutes at 450 *g* and recovering the pellet.

Isolation of Fractions

Breis were made by repeatedly forcing living animals through a syringe. Cilia and trichocysts were prepared by the salt-alcohol method of Preer and Preer (7). This method avoids the use of detergents (*cf.* 10) and the disruption of cells. The cilia were concentrated by centrifugation at 25,000 *g* for 5 minutes at 0°C., and both trichocysts and cilia were resuspended in buffered saline (0.9 per cent (*w/v*) NaCl and 0.01 M sodium phosphate pH 7.0). To isolate mitochondria, the pellet of whole animals following loss of cilia and trichocysts was used. The pellet was recovered after centrifugation in 120 ml. pear-shaped centrifuge tubes at 700 *g* (maximum speed) in an International oil testing centrifuge for 1 minute at room temperature. The pellet was then homogenized with a teflon pestle in 2 ml. of saline in a 30 ml. glass cylinder for 2 minutes, and the resulting brei centrifuged for 5 minutes at 1000 *g* at 0°C. in the high speed head of a PR-2 International refrigerated centrifuge. The supernatant was decanted and centrifuged at 4000 *g* for an additional 5 minutes. The precipitate, resuspended in buffered saline, comprised the mitochondria fraction. The supernatant of this preparation, following centrifugation at 25,000 *g* for 15 minutes, yielded a precipitate which was termed the small granule fraction and was suspended in buffered saline. All fractions were stored at −20°C. following isolation. No further extraction or purification was carried out. Generally 6×10^6 paramecia were fractionated and the antigen solutions brought up to a volume of 1 ml.

Characterization and Identification of Fractions

The appearance of the cilia and trichocysts under the phase-contrast microscope was essentially as described by Preer and Preer (7). Of the two fractions, the cilia preparation was more homogeneous with few other particles or debris. The trichocyst fraction, however, always contained a small proportion of cilia. Both cilia and trichocysts generally appeared unfragmented immediately after isolation.

Mitochondria were identified by their speed of sedimentation, size, and shape in cold saline, stainability with fast green following Harman's procedure (11), and ability to oxidize reduced horse heart cytochrome *c*. (This latter criterion, however, could only be very irregularly reproduced.) The yield of these particles appeared to be considerably higher than that achieved with the procedure described by Preer and Preer (7). The particles were regularly spherical, except immediately after isolation, differing from the rod shapes observed when isolation is carried out in raffinose. Their diameter, about 1 μ, was somewhat less than that of spherical mitochondria swollen in saline, prepared from animals not treated with alcohol.

The small granule fraction consisted of spheres of about 0.5 μ in diameter that remained remarkably constant in size and number in volumes of 1 ml. or less at room temperature. They did not stain with fast green (11).

Antisera

The antisera used to study these fractions were obtained following the injection of homogenates of whole animals or fractions into rabbits. The usual course was three injections about 1 week apart, each consisting of 2.5 ml. of Freund's adjuvant (12) (Difco Laboratories, Detroit, Michigan) blended with 2.5 ml. of the antigen prepared from about 2×10^6 paramecia and injected subcutaneously. Postimmunization bleedings were from the marginal ear vein generally 1 week after the first injection and twice a week thereafter. Sera thus obtained, together with preimmune sera, were stored in 3 to 4 aliquots without preservative at -20°C.

Gel Diffusion Methods

Antigens were analyzed primarily by methods based on the suspension of an antigen-antibody precipitate in a gel, the location of the precipitate being a function of the concentrations and diffusion coefficients of the antigen and antibody and other less specific factors. Diffusion through gels permits the simultaneous study of several antigens in a mixture, yielding both qualitative and quantitative results.

Two techniques were used: the tube method of Oakley and Fulthorpe (13) as modified by Preer (14), and the Petri dish method described by Ouchterlony (15, 16, 17). In the tube method a tube of 1.7 mm. inside diameter is coated with 0.1 per cent agar and flame sealed at one end. A 4 mm. column of one reactant, generally antiserum, is then placed in the bottom of the tube. Over the serum is layered 0.6 per cent fluid agar (containing 0.01 per cent merthiolate and 0.5 M glycine) at 50°C. After solidification in the cold, the antigen solution is added and the tube sealed with pyseal (Fisher Scientific Co., New York City). A tuberculin syringe fitted with a 2 inch 26 gauge needle is used for loading. The exact heights and volumes of antiserum and antigen solutions are not critical (0.01 ml. of each is sufficient), but for optimal resolution of bands the agar should be about 4 to 5 mm. high.

For the Ouchterlony plates the various patterns of wells were cut from the agar (same composition as for the tubes) with cork borers. All of the patterns had one feature in common: the borders of antigen and antiserum wells were within 6 mm. of each other. Wells were never refilled, and evaporation was minimized by keeping the covered dishes at 8°C. or in moist chambers at 17°C. Within 4 days most, and usually all, of the bands had developed. A photographic record was then made of the unfixed plates.

Absorption Experiments

To remove selectively an antibody from a serum, mixtures of the antiserum and the solution of antigens (total volume of 0.6 ml. or less) were made in 12 ml. conical centrifuge tubes. The stoppered tubes were incubated at 37°C. for 1 hour and then placed at 8°C. for 18 to 72 hours. The supernatant collected following centrifugation in the low speed head of the International PR-2 (220 *g*, 1 hour) was then assayed in gel for changes in antibody content. In testing for antibody excess in tubes, the absorbed serum was placed as the bottom layer and more dilute agar than the usual 0.6 per cent added. For antigen excess tests, the bottom layer was generally undiluted serum.

Immobilization Tests

When paramecia are placed in antisera prepared against whole or homogenized paramecia, they will be immobilized if they are of the same or a related serotype as that of the injected animals, or will be unaffected if they belong to another non-cross-reacting serotype. The serotype of a culture was determined by placing samples into the appropriate dilutions of different specific antisera. The immobilization titer of a serum was determined by making a dilution series of the serum with Dryl solution (18) or a 10 per cent Ringer's solution and noting the lowest concentration that will immobilize animals of a particular serotype in 2 hours at 27°C. For a more complete description of serotype determination and serum titration see Sonneborn (19).

RESULTS

Localization of the Immobilization Antigens

Beale and Kacser (6) and Preer and Preer (7) have shown by methods other than those used here that the immobilizing antigen is distributed widely

TABLE I

Immobilization Titers of Sera against Entire and Fractionated Paramecia

Serum Rf#	Stock or strain	Serotype	Preparation of antigen	Immobilization titers against serotypes of animals used as source of antigen
1	*d*30-1	C	brei	1:400
3	*d*30-1	G	brei	1:400
4	*d*30-1	G	brei	1:400
6	*d*30-1	A	brei	1:200
7	*d*30-1	C	brei	1:200
8	*d*30-1	C	brei	1:400
9	7	C	brei	1:200
10	7	C	brei	1:800
11	*d*28-1	E	brei	1:400
14	hy-brid	C	brei	1:200
16	*d*30-1	C	det.* small granules	1:50
17	*d*30-1	C	det. small granules	1:100
18	*d*28-1	E	det. small granules	1:12.5
21	197	unknown	brei	1:800
23	7	G	det. small granules	1:12.5
27	7	C	det. small granules	1:25
29	7	C	trichocysts	1:200
30	*d*30-1	C	mitochon-dria	1:200
31	*d*30-1	C	trichocysts	1:400
32	*d*30-1	C	mitochon-dria	1:25
33	*d*30-1	C	trichocysts	1:200
34	7	C	det. small granules	1:12.5
35	*d*30-1	C	det. small granules	1:12.5
40	72	unknown	trichocysts	1:800
41	72	unknown	cilia	1:800
42	*d*28-1	E	trichocysts	1:800
44	*d*28-1	E	cilia	1:800

* det. = detergent

in a *Paramecium*, most of it, however, being in the cilia and pellicle. It was of interest then to compare the immobilization titers of antisera prepared against the different antigens–breis and isolated particles. 27 sera were analyzed: 11 prepared against breis of entire paramecia, 2 against cilia, 5 against trichocysts, 2 against mitochondria, and 7 against detergent-prepared small granules (description of detergent method in preparation). Only sera with precipitating antibodies in relatively high concentration were used.

Because the antibody response may amplify the occurrence of antigens present in small amounts, especially when the antigen is incorporated in an adjuvant, the immobilization titers of sera against different organelles are sensitive indicators for the unique localization of immobilization antigens. From the data in Table I, it can be seen that mitochondria antisera and detergent-prepared small particle antisera had the lowest immobilization titers, while trichocyst and cilia antisera, aside from brei antisera, had the highest titers. (Since all trichocyst preparations are contaminated to some extent with cilia, it may be that the immobilizing titer of the trichocyst antisera is due to these cilia rather than an antigen shared by trichocysts and cilia.) In contrast, only late bleedings from the two rabbits injected with mitochondria possessed any significant immobilization titer, as would be expected were small amounts of contaminating antigen present.

Thus it appears that mitochondria and detergent particles are fairly free of immobilizing antigen, either as contaminant or intrinsic antigen, while cilia and trichocysts (the pellicle was not tested) have the antigen in abundance.

Relationship of Spurs to Cross-Reactions of Immobilization Antigens

Bands in a plate of agar that form in adjacent areas continue to extend lengthwise with time as more antigen and antibody diffuse and react in concentrations that allow a visible precipitate to form. There may be opportunity for two precipitates, formed separately by reactants originating from neighboring wells, to impinge on each other. If the precipitates have no reactants related to each other, the precipitates will not act as barriers and the bands will cross each other.

However, if two antigens diffusing against a single serum from separated wells cannot be distinguished by the antibodies in the serum, a "V" will be formed by the fusion of the two bands. And, lastly, solutions of two different antigens that are sufficiently alike may form a "spur" or extension of one of the arms of a V when both of the antigens diffuse against a serum prepared against

one of them. (See (17) for a more detailed account of these and other possible reactions.)

There are data from other systems for the equivalence of spurs with incompletely cross-reacting antigens—*e.g.*, hen and duck albumin (21), and various myoglobins (22). In a study utilizing a hapten conjugated to gamma globulin several procedures were followed to validate the equation of a spur with cross-reactivity (23). These same procedures were used with solubilized immobilization antigens, made either by extracting cilia overnight in saline or homogenizing whole animals and using the supernatant obtained after centrifugation (24).

Although cross-reacting antigens when diffused against serum prepared against one of them will generally form a spur, the converse of this is not true; all spurs are not due to cross-reactions. At least three other situations may give rise to spurs: (*a*) The same antigen may be present in widely differing concentrations in neighboring wells; (*b*) two unrelated antigens, one of which is not present in sufficiently high concentration to form a band penetrating the other precipitate, may give the appearance of a spur; (*c*) one well may have two antigens, only one of which is in common with he single antigen present in the adjacent well. The unrelated antigen may form a band overlying the first antigen, the latter completely fusing with the neighboring precipitate to form a V but with an extension of one arm due to the overlying unrelated antigen.

If the antigen solution forming the spur is termed the homologous antigen, then when absorptions are carried out with sufficient quantities of the homologous antigen and the absorbed serum tested with the two antigen solutions, precipitates would not form except in the second instance, where two unrelated antigens are involved. However, when the heterologous antigen solution is used as absorbent, bands will form with the homologous antigen solution except in the first instance. To distinguish between spurs due to overlapping antigens and to cross-reactions, not only is the presence of a band significant, but of prime importance is the position of the band, position being a measure of antibody or antigen concentration (14). With true cross-reactions, the band remaining will be less intense (as would also be true with overlapping bands) and will alter its position towards the serum well, (which would not be true in an instance of overlapping bands), indicating a removal of cross-reacting antibody. Thus, by noting whether a band forms with only one of the antigens following the appropriate absorption and

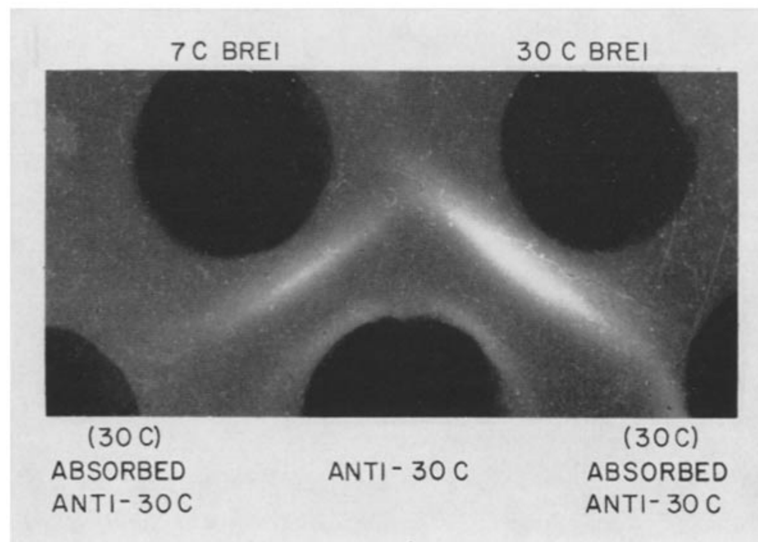

FIGURE 1

Comparison of diffusion patterns of unabsorbed anti-30 C serum and the same serum after absorption with homologous antigen (30 C). The middle well in the bottom row contains the control (unabsorbed 30 C antiserum). × 3.

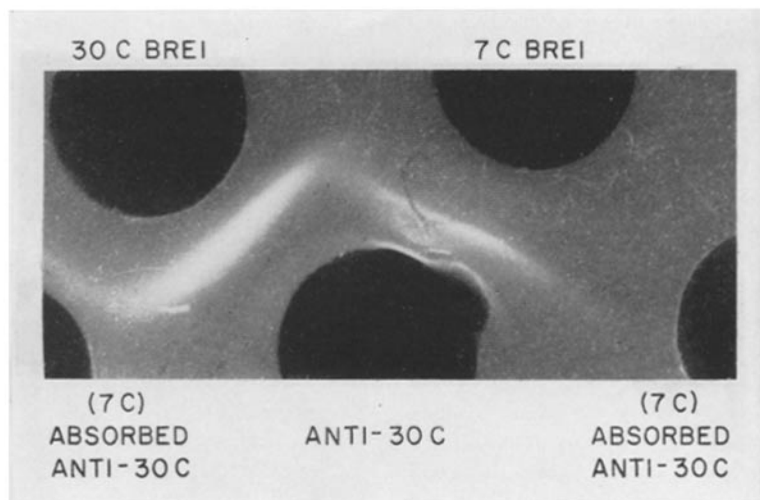

FIGURE 2

Comparison of diffusion patterns of unabsorbed anti-30 C serum and the same serum after absorption with heterologous cross-reacting antigen (7 C). The middle well in the bottom row contains the control (unabsorbed 30 C antiserum). × 3.

by further noting any change in a band's position, spurs and true cross-reactions can be equated.

When the C immobilization antigens were extracted from stock 7 and derived line *d*30-1 (see (24) for identification of antigens in gel) and diffused against antisera prepared against one stock, the expected spur was observed (Fig. 1). When the homologous antigen was used as absorbent, all antibody was removed; when heterologous antigen was used, only the heterologous band was completely removed. The antibodies specific for the homologous antigens were still present, as evidenced by the formation of a band (Fig. 2). The band, it should be noted, has shifted towards the serum well as would be predicted. More precise estimates of the change in location were made with the aid of gel diffusion in tubes. Thus, with antigens known to cross-react by *in vivo* immobilization tests, spurs have been shown to reveal cross-reactivity when the same antigens in solution were used.

Relationships among Fractions

There was no fraction that could be relied on to form the same number of bands when repeated preparations of the fraction were made. Homogenates of whole cells, in contrast, were quite uniform in the number and quantity of antigens detectable (Fig. 3). In over 50 plates representing more than 100 repeated preparations, rarely was a single antigen completely lacking. The lack of good reproducibility with fractions makes interpretation of data difficult, particularly if presence or absence of a band is to be used as a criterion. Although repeated, as opposed to sporadic, failure to demonstrate a particular band with a fraction of course can be meaningful, in these studies this criterion was not relied upon.

Several immunological studies of particles (*e.g.*, 2, 3), including the present ones, have shown many antigens to be shared among particles. Although the possibility of adsorption during the isolation procedure to account for this cannot be completely eliminated, other evidence points to the uniqueness of some of the antigens of the fraction isolated. As noted above, not all fractions elicit immobilization antibodies when injected and so, at least with respect to the immobilization antigen, the fractions are dissimilar. Also indicative of the uniqueness of the isolated particles is the fact that the cross-reactions described below occurred only with two of the fractions in any particular experiment. If contamination and adsorption were widespread, then cross-reactivity should be common among all particles simultaneously.

Emphasis has been placed on the utility of reactions in gels to indicate cross-reactivity because this criterion provides a simple and straightforward

test for identity, similarity, or complete distinction between particles. The use of cross-reactivity as a criterion helps overcome the disadvantage of variability in duplicate experiments because only a pair of bands already present in a plate is being compared. Cross-reactivity can also provide important evidence for the lack of contamination of a fraction due to adsorption of a soluble antigen native to a second fraction. Antigens forming true spurs as a result of cross-reactions must be different and thus probably reflect differences between particles from which they are extracted.

Paramecia fractions were compared in gel against antiparamecia sera of all types. Although spurs are usually absent, and the bands show either complete separation or complete identity, occasionally spurs are undoubtedly found. These occurred between mitochondria and cilia, between cilia and trichocysts, and between mitochondria and trichocysts. Fig. 4 shows an instance of one such spur between mitochondria and trichocyst antigens, with the spur forming as a continuation of the homologous precipitate (mitochondria antigen and serum from a rabbit injected with mitochondria).

With *Paramecium* the presence of these spurs need not necessarily mean that each of these sets of particulates possess hitherto unsuspected related antigens. At least one other antigen is known to exist *in vivo* that would show this cross-reactivity, the immobilization antigen, and this has been demonstrated to exhibit spurs in gel (see above). That the spurs described here are not due to the immobilization antigen is shown by the fact that the immobilization antigens that cross-react are either found in different stocks or when present in a single stock are never expressed simultaneously (8, 20). The spurs here were found within a single stock between fractions prepared at the same time. Also the presence of spurs often has no correlation with the kind of immobilization antibodies in the sera used.

Again the multiplicity of cross-reactions may be illusory, the same antigens participating in all fractions. That this was not so was shown by instances in which a spur with two of the fractions formed in a direction indicating a certain fraction as acting homologously, while simultaneously the "homologous" fraction indicated a "non-homologous" cross-reaction with yet another fraction. Also, a rearrangement of wells can demonstrate that the same antigens are not involved.

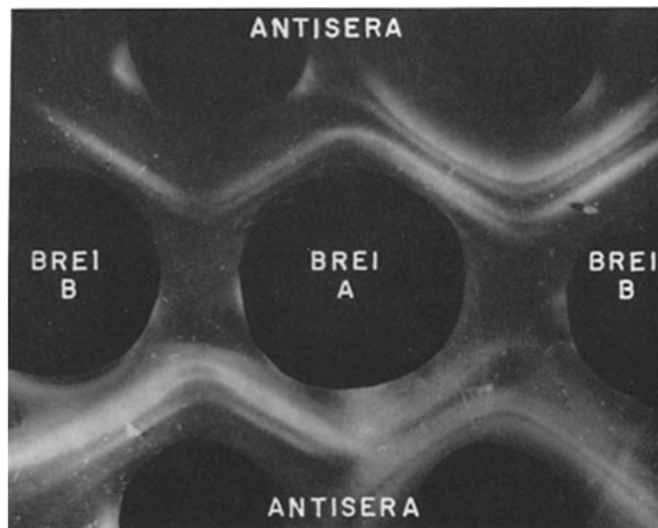

FIGURE 3

Comparison of breis. Each of the four wells of the top and bottom rows is filled with a different antiserum. In the middle row the two end wells are filled with the same antigen solution, and the middle one with another preparation of the same stock. Even with the large numbers of bands formed, there is excellent reproducibility between the two homogenate preparations. × 3.

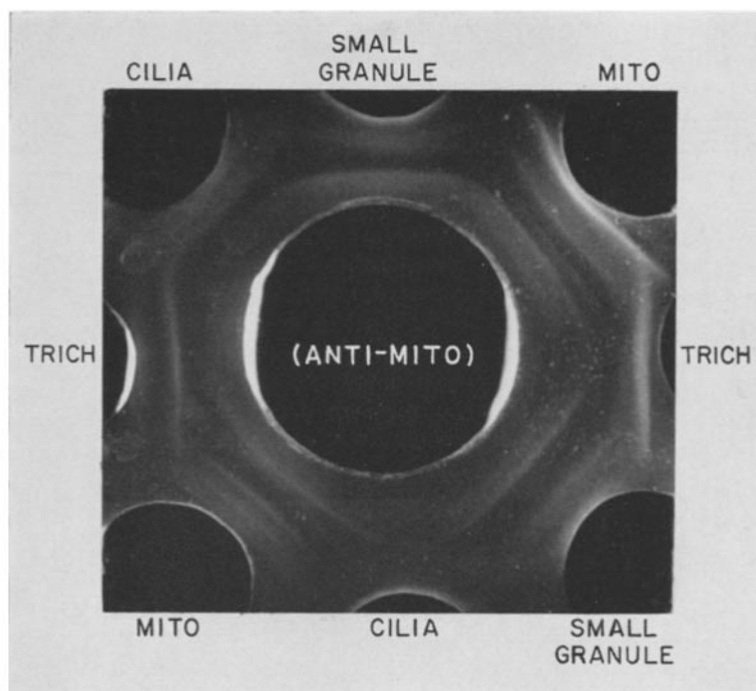

FIGURE 4

Comparison of different fractions prepared from the same culture. The central well is filled with Rf #30. The spur between mitochondria and trichocysts in the wells on the right is prominent. The duplicate wells, on the left, show the spur much fainter. This illustrates the variations to be expected in duplication even within the same plate. × 3.

Variations of Mitochondrial and Small Particle Antigens within a Clone

Although contamination, non-specific adsorption, etc. are often insurmountable barriers to drawing conclusions from studies with disrupted cells, certain experiments may be carried out that may safely ignore these hazards. One of these is a comparison of the antigens of the same fraction isolated from the same stock or from different stocks at different times, so long as the same method of preparation is strictly adhered to. Thus the danger of heterogeneity due to contamination is unimportant if the same degree of contamination occurs in replicate trials.

If the antigens extracted from, say, mitochondria prepared at different times from the same vegetatively reproducing strain, are constant in their specificity, then when a series of such successively made preparations are compared in gel only complete fusion of bands should be found. However, if alterations in specificity were to occur, then at least two kinds of gel diffusion experiments should possibly reflect these shifts. First of all, when two different strains are compared using a serum made against only one of the strains, occasionally a spur should be observed which is an extension of the band formed with a strain *not* injected; *i.e.*, the injected strain would not react in the expected homologous fashion. Secondly, when different preparations of the *same* strain are compared spurs should be seen.

Of special interest, therefore, was the original observation that there seemed to be little correlation between the stock injected and whether, when the same stock was used to prepare test antigens, these antigens reacted to indicate homology—*i.e.*, to form a spur along the "homologous" front when compared with other antigens. Thus, often the antigens of a stock other than that injected would form more bands and, more significantly, when forming spurs would manifest the spur as a homologous extension, while antigens from a clone derived from the originally injected culture would react in a heterologous manner. Absorption experiments

confirmed that the spurs formed were indeed the result of cross-reactions.

These transformations were not restricted to mitochondria, but also occurred with another fraction, the small granules, and generally quite independent of each other. Thus, a spur with one fraction did not signal that antigens from other fractions of the same culture would similarly cross-react. Again, like the immobilization serotypes, these antigenic shifts were not permanent, but reversible, the spurs disappearing when later preparations were employed. This lability also speaks against the antigenic changes being the result of gene mutations.

Additional evidence for antigenic transformation came from comparison in gel of mitochondrial and small particle antigens from the same strain grown at two temperatures, 17° and 27°, and 17° and 31°C. When these soluble antigens, prepared at different times, were diffused against mitochondria antiserum or against a detergent-particle antiserum, spurs formed that could be specifically removed by the appropriate absorption (Fig. 5). These intraclonal spurs formed very rarely (only three such instances being observed of more than 10,000 opportunities).

The spurs described could possibly be due to differences induced by the isolation and extraction procedures, although the same procedure was followed as closely as possible in making replicate

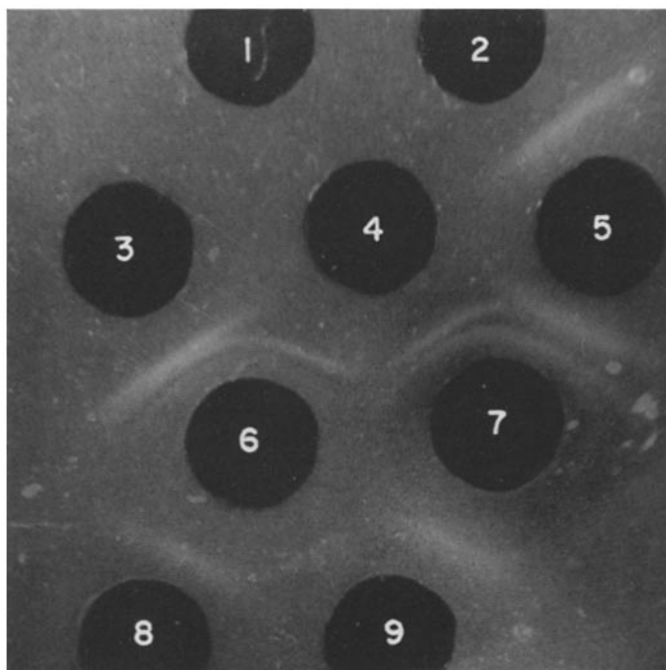

FIGURE 5

Comparison of small granule antigens extracted from two cultures of the same clone (stock 7) harvested 2 months apart.

Well *1*, supernatant of serum absorbed with small granule antigen 9/16/59; well *2*, supernatant of serum absorbed with small granule antigen 7/9/59; well *3*, small granule preparation of 9/16/59; well *4*, small granule preparation of 7/9/59; well *5*, small granule preparation of 9/16/59; well *6*, unabsorbed serum (diluted 1/2); well *7*, unabsorbed serum; well *8*, preparation of 9/16/59 (diluted 1/2); well *9*, preparation of 7/9/59 (diluted 1/2).

The spur has formed so that the preparation of 9/16/59 appears as homologous (wells *3* and *5*). This is even evident with the diluted antigen (well *8*). As expected, only when the serum is absorbed with the "heterologous" antigen (well *2*) is there antibody remaining. The quantitative loss of antibody (as measured by change in band position) was followed in small tubes. (The "split" in the band between wells *3* and *6* is probably an artifact of concentration. None of the other bands show it). × 3.

preparations of antigens. However, when a culture was divided into several aliquots and fresh preparations of fractions were prepared separately, spurs were never found in Ouchterlony plates. Similarly, when preparations of several cultures were carried as far as the salt-alcohol step of preparation and stored at $-20°C$. and then aliquots removed at weekly intervals and fractions isolated, no spurs were observed between aliquots. Furthermore, when immobilization antigens from breis of the same serotype were extracted at different times the cross-reactions in agar patterns were always formed as expected—antigens that cross-reacted *in vivo* formed spurs; non-cross-reacting antigens did not. Also, if spurs were more often than not artifacts of preparation, there should be no difference in frequency of spurs when different strains or when preparations from different cultures of the same strain are compared. However, spurs with the latter are rare events, while with the former they are not (unpublished data). These findings taken together suggest that the spurs observed in other experiments are neither artifacts of gel diffusion nor artifacts induced by the method of preparation of the antigens.

DISCUSSION

From an immunological analysis of some of the more rapidly sedimenting particles of a cell, it has been shown that different fractions of the particles probably possess some soluble antigens which have related, but not identical, specific groups. It is not known whether these antigens are structural components or represent molecules with enzymatic functions (or are both). Nonetheless a relationship may exist between particles not evident from purely morphological considerations—*e.g.*, similarity of antigens present in such morphologically distinct structures as trichocysts and mitochondria. Furthermore, there is evidence for variations within clones of cells in the composition of a universal cell constituent, the mitochondria. This evidence comes primarily from the presence in agar plates of spur formation between antibodies diffusing against solutions of mitochondrial antigens, supported by absorption experiments and tests of the supernatants in tubes and plates. Indeed, it is possible, although quite difficult, to study cross-reactions between individual antigens in a complex mixture of antigens without reference to spurs. This would entail careful measurements of band position and intensity in tubes as well as the positions of all bands formed at immunological equivalence (as a rough guide to identification) and following absorption. If only a single band is present, then such analysis becomes practicable. However, the relationships of the mitochondrial antigens described probably could not have been eludicated with reference only to double diffusion in tubes without extensive purification. In any event it would seem that the immunological approach can be a sensitive and flexible tool to determine changes and relationships of populations of intracellular organelles.

Of some possible significance is the striking parallel to be found between mitochondrial antigenic variation and the transformation of the immobilization serotypes. Unfortunately genetic analysis must be postponed until a means of controlling the mitochondrial shifts as well as a way of utilizing antigen extracted from a relatively few number of animals can be found. However, simply by analogy, and consistent with what has been described above, it appears that the antigens of many particulates of the cell acquire different specificities during the existence of a cell. The alterations are readily reversible (unlike genic changes) and probably induced by environmental changes. Beale (25) has postulated the existence of different cytoplasmic states as calling forth the expression of the genes determining the immobilization antigens. To apply the concept here, possibly a number of independent cytoplasmic states would, by extrapolation, be needed to account for the several transformations—mitochondrial, ciliary, small granules, etc.

Again, with the immobilization antigens in mind, it can be understood why cross-reactions within a stock are rarely found. Presumably, different loci are controlling the specificity of the different antigens within a strain, with the activity of only one locus at a time being expressed. Structural similarities would be expected to be rare, or at any rate more infrequent, between antigens determined by different loci than when the antigens are determined by different alleles at the same locus. Supporting this view are preliminary experiments in which many more instances of spurs are found in comparisons of antigens from different strains. Here both cross-reactions due to interloci and interallelic differences would be expected to occur. However, even in these experiments spurs are not a frequent event. A plausible explanation for this rarity is that generally 6×10^6 cells must be used

to yield sufficient antigen for analysis, and assuming at least 10,000 of each kind of particulate per cell (7) it would not be expected that any shift other than a unilateral one involving a considerable proportion of all the particles would be detected. Of course, the rarity of cross-reactions may truly be evidence for intrinsic antigenic stability.

The significance of the mitochondrial variations observed depends in part on what antigenic variations may mean with respect to function. It could be argued that if rabbit and *Paramecium* mitochondria perform similar functions and the antibody-forming sites of the rabbit have been exposed to its own mitochondria, then antibodies formed against another species' mitochondria must be against non-functional species-specific regions of the mitochondria. On the other hand, several instances have been reported of inactivation of respiratory enzymes by specific antibodies, although whether this occurs as a result of steric hindrance or actual combination with active sites is not known (26, 27).

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