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Reaction of Chick Embryo Cells Infected with Leukosis Strain MC29 Virus with Fluorescein-Labeled Antibody

ROBERT B. FRITZ, A. J. LANGLOIS, AND J. W. BEARD
Department of Surgery, Duke University Medical Center, Durham, North Carolina 27706

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Immune serum was prepared in the rabbit with BAI strain A leukosis virus isolated by centrifugal fractionation from the plasma of chickens with myeloblastic leukemia and further purified on a potassium tartrate gradient. Antibody to group-specific antigen was demonstrated in the serum by immunoelectrophoresis and immunodiffusion. Fluorescein-conjugated serum was used unabsorbed and absorbed with chick cells for study of acetone-fixed chick embryo cells uninfected or infected with strain MC29 avian leukosis virus. With unabsorbed serum, large numbers of cytoplasmic particles stained in a few cells within 2 hr after exposure to virus, and the cell number increased greatly in 24 hr. Absorption of the serum abolished the early reaction. Staining with absorbed serum was delayed until about 14 hr after culture exposure to virus, but essentially all cells were stained within 72 hr at the time when all cells were morphologically altered. Differences between the responses to unabsorbed and absorbed serum suggested cytoplasmic formation or concentration of chick tissue antigen similar to that incorporated in leukosis virus particles. The characteristics of staining with absorbed serum were similar to those observed by others in analogous studies with avian tumor viruses.

Strain MC29 (12) avian leukosis virus induces chiefly (12, 19) myeloid hematopoietic tissue tumors or diffuse growths of myelocytes in the chicken. The virus and growths contain group specific (GS; 11) and other antigens common to the avian virus-tumor complex as demonstrated (9) in cross-reactions by gel immuno-precipitation, neutralization, and complement fixation tests.

Strain MC29 likewise induces infection of chick embryo cells (CEC) and liberation of virus detectable within 12 to 18 hr (15) by focus formation assay (14). CEC thus infected also produce GS and other viral antigens (9), but, unlike CEC exposed to other leukosis strains, exhibit rapid alteration of morphology (4, 10, 16). Change, detectable as early as 48 hr after infection, appears to involve all of the cells in periods as brief as 72 to 80 hr (2).

These and other aspects of culture behavior suggested high incidence of, if not uniform, infection and change in the individuals of the initial chick embryo cell (CEC) population induced by MC29 virus. In contrast, in other in vitro avian tumor virus systems, low levels of response to virus exposure were indicated by a variety of criteria (21, 25), principally in the low rate of change in cell morphology and the results of infectious-center tests. However, high incidence though delayed response to Rous sarcoma virus was observed with fluorescein-conjugated antibody (26). It was thus of interest to determine whether the labeled-antibody reaction of strain MC29-infected and altered cells differed quantitatively or qualitatively from that with cells infected with other avian tumor agents. The experiments for this purpose were made with serum from rabbits immunized with BAI strain A leukosis virus which is immunologically closely related to the strain MC29 agent. Application of absorbed serum revealed antigens appearing in the cells as those reported with the other tumor agents. Unabsorbed serum, however, disclosed a different intracellular virus-specific antigen occurring much earlier than that ordinarily observed. This report describes the investigations with CEC cultures infected with relatively high MC29 virus multiplicity.

MATERIALS AND METHODS

Preparation of cultures. CEC in primary cultures (2) from embryos free of resistance-inducing factor (RIF; 22) were taken up with 0.05% trypsin, sedi-

mented, and then suspended in growth medium in a concentration of 10^5 cells/ml. Sykes-Moore culture chambers (24) assembled loosely with one cover glass and "O" ring gasket were autoclaved in glass petri dishes. The gasket was tightened against the glass, and 8×10^4 cells (0.8 ml of suspension) were added to each chamber. The open chambers in petri dishes were incubated at 38.5 C in a CO_2-humidified incubator. After 24 hr the cell layer was washed with 0.5 ml of phosphate-buffered saline (PBS) and inoculated with 0.1 ml of virus [1.0×10^5 focus-forming units (FFU) or 5.6×10^7 virus particles determined by direct count with the electron microscope (23)]. After 30 min of incubation at 38.5 C, the cells were washed twice with 0.5 ml of PBS, and 0.8 ml of medium was added. Parallel control cultures were kept without virus. Cultures terminated at 2-hr intervals during the first 20 hr after infection and, at 24, 36, 48, 60, and 72 hr, thereafter, were washed with PBS, fixed for 8 min in cold acetone (−78 C), and stored at −20 C. Corresponding culture fluids were stored at −78 C for virus assay (14).

Antigen. BAI strain A (myeloblastosis) virus was obtained from the plasma of chickens with leukemia by three cycles of differential centrifugation and banded on a 2 to 40% (w/v) potassium tartrate gradient (3). Virus from the gradient was sedimented by centrifugation, resuspended in 1 ml of PBS, and emulsified with an equal volume of Freund's incomplete adjuvant. Inoculations of 0.5 ml were made into the hind foot pads of two rabbits, approximately 10^{11} virus particles per animal, and repeated in the same way at monthly intervals. Blood was taken from the median artery of the ear at weekly intervals. High-titer hamster GS immune serum was generously provided by R. M. Dougherty.

Preparation and conjugation of antibody globulin. Immune globulins were precipitated at room temperature with 2 volumes of 27% (w/v) sodium sulfate to 1 volume of serum. The globulins dissolved in distilled water were reprecipitated with an equal volume of 27% sodium sulfate. This procedure was repeated once more. The final precipitate redissolved in a minimal amount of distilled water was dialyzed against several 2-liter changes of phosphate buffer ($\mu = 0.02$, pH 5.4). Precipitate forming during dialysis was discarded, and the supernatant fluids were lyophilized. This material was entirely gamma globulin as characterized by immunoelectrophoresis with sheep-antirabbit serum.

Portions (20 mg) of globulin were conjugated with fluorescein isothiocyanate (BBL Division of Bio-Quest, Cockeysville, Md.) by dialysis (5) for 24 hr at 4 C. Unreacted dye was removed with a Sephadex G-50 column (1.5 × 8 cm; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), and the conjugate was eluted with PBS. The material immediately following the void volume was collected, dialyzed for 24 hr against several changes of PBS, centrifuged, divided into small portions, and stored at −20 C. For certain experiments, 1 ml of the conjugate was absorbed for 1 hr at 37 C and then for 12 hr at 4 C with 1×10^8 uninfected frozen and thawed CEC.

Immunoelectrophoretic and immunodiffusion analysis of antisera. Antisera for conjugation with fluorescein isothiocyanate were tested by microimmunoelectrophoresis for reactivity with BAI strain A and strain MC29 virus disrupted with 1% sodium deoxycholate (SDC). Virus antigen migrated for 50 min in a field of 3 v per cm in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 8.0) in 1% noble agar (Difco). Troughs were cut in the agar, and the immune serum was added. After 24 to 72 hr of incubation at 37 C, the unstained agar slides were photographed with indirect illumination. Immunodiffusion patterns were obtained as described in a previous paper (9).

Staining with conjugated globulins. Fixed normal, MC29-infected, or MC29-altered CEC washed with PBS were treated with one drop of the conjugate. The cover slips were incubated at 37 C for 60 min in a moist atmosphere, washed four times for 5 min with 30 ml of PBS, and mounted in 9:1 glycerol-PBS for examination with a Leitz fluorescence microscope. As further controls, infected CEC were incubated with normal rabbit serum or unlabeled immune serum before application of the conjugate.

RESULTS

The gamma globulin fraction of the rabbit immune serum before conjugation contained approximately 12% BAI strain A virus antibody as determined by the quantitative precipitin reaction with native virus (8). An additional 4 to 5% of the serum globulin was precipitable by GS internal antigen. In Fig. 1A, the immunoelectrophoretic pattern obtained with this serum and SDC-disrupted BAI strain A virus is com-

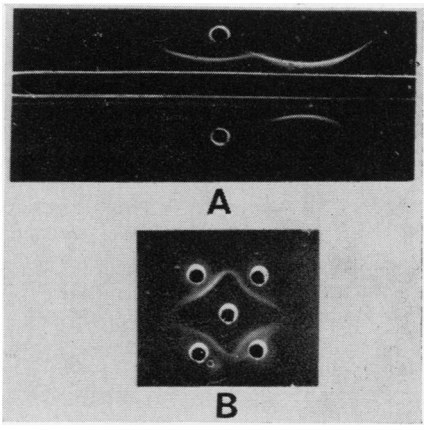

FIG. 1. (*A*) *Immunoelectrophoretic patterns (anode to the left) of the reactions of unabsorbed anti-BAI strain A virus immune serum from the rabbit (trough) with SDC-treated BAI strain A (upper well) and strain MC29 (lower well) viruses.* (*B*) *Gel precipitin reactions of unabsorbed rabbit anti-BAI strain A virus immune serum (upper left and lower right wells) and hamster anti-group-specific immune serum (upper right and lower left wells) with sodium-deoxycholate-treated strain MC29 virus (central well).*

pared with that of tissue-culture-grown strain MC29 virus purified and disrupted in the same way. The component moving most rapidly toward the cathode was a constituent of GS antigen (R. Fritz, *unpublished data*); no studies were made to identify the antigen yielding the precipitin line at the point of origin. The gel diffusion pattern of Fig. 1B gave additional evidence of GS antibody in this immune serum which was demonstrable by the line of identity observed between hamster GS antiserum and the rabbit immune serum tested against disrupted strain MC29 virus.

In the studies of virus antigens within the cells, labeled serum was used prior to and subsequent to absorption with normal CEC. Uninfected CEC monolayers were not stained by unabsorbed serum (Fig. 2A), but a small proportion of infected cells showed fluorescence in small discrete areas within the cytoplasm as early as 2 hr after exposure to MC29 virus (Fig. 2B). The proportion of cells stained with unabsorbed conjugated serum increased with increasing time, and, in addition, antigen was concentrated at the periphery of some cells at 12 hr after infection (Fig. 2C). At 48 hr after infection, much of the fluorescence was associated with long threadlike processes or filapodia extending from the cells (Fig. 2D). Such staining was diminished upon preincubation of cover slips with unlabeled immune serum, but not by preincubation with normal serum.

Absorption of the conjugate with a large excess of normal CEC completely abolished the early staining reactions, and other specific fluorescence did not appear until about 14 hr after exposure to the virus (Fig. 3A). At this time, staining was very faint and distributed throughout the cell cytoplasm, and the proportion of stained cells was very low. Cells fixed 36 and 48 hr (Fig. 3B and 3C) after infection exhibited fluorescence primarily in the filiform processes as did those treated with the unabsorbed conjugate (Fig. 2D). At 72 hr after infection, the cells exhibited fluorescence very strongly at the membrane (Fig. 3D).

Staining of nuclear components was not observed with either unabsorbed or absorbed conjugated serum.

Long-term cultures were tested for virus antigen at 13, 35, and 56 days after infection. Strong fluorescence was observed in the 13-day and 35-day cultures (Fig. 4A and 4B). The 56-day culture, however, exhibited very weak cytoplasmic fluorescence (Fig. 4C). At this time, the cultures in this series were not in vigorous growth. All cells in the long-term cultures, 13 to 56 days infection, stained with the conjugate.

Fluids collected from the CEC cultures were assayed for infectious virus (14). As may be seen in Table 1, newly synthesized infectious virus appeared 12 to 14 hr after infection. This corresponded closely to the time of appearance of the antigen staining with adsorbed immune serum. The foci produced by the 2- to 10-hr culture fluids were due principally to residual virus from the inoculum.

DISCUSSION

Previous studies of the response of avian tumor virus infected cells to fluorescein-labeled antibodies demonstrated much variation in the cellular distribution and localization of antigen (13, 17, 18, 20, 27). Although the cell type and infecting virus strain might be largely responsible for such variation, the source of immune serum could likewise be an important factor. Immune sera usually employed were from the chicken or hamster, and, with respect to the total spectrum of virion antigens, both were incomplete. Sera from the chicken ordinarily contain only antibody to virus type-specific antigen (1), whereas those from hamsters with Schmitt-Ruppin virus tumors appear to react only with the avian-leukosis GS antigens, and not with the virus surface antigens (6, 13). In addition, recent work (1; F. K. Roth and R. M. Dougherty, Bacteriol. Proc., p. 149, 1967) has demonstrated that hamster GS immune serum recognizes several antigens in both avian leukosis-sarcoma viruses and infected cells. Because of limited individual specificities, it is thus likely that none of these sera could react with all viral antigens within the infected cells, and, furthermore, the multiple specificity of hamster anti-GS immune serum could lead to variations in the staining patterns of the cells.

For the present studies, antibody was prepared in rabbits with density-gradient purified BAI strain A virus from plasma of chickens with myeloblastic leukemia. This agent was used instead of MC29 because (i) it could be obtained from plasma in high concentration with little nonviral contaminant (3), which would have been difficult to have achieved with MC29 virus occurring only in low concentration in tissue culture fluid (15); (ii) it induces strong cross-neutralizing antibodies reactive with strain MC29 (9); and (iii) immunization of rabbits with BAI strain A produces potent anti-GS antibody (6). Immune serum obtained by immunization of rabbits with virus concentrated by conventional centrifugation produces potent antiserum against particle-coat antigens but not against GS antigen (6). Antiserum against GS antigen of the

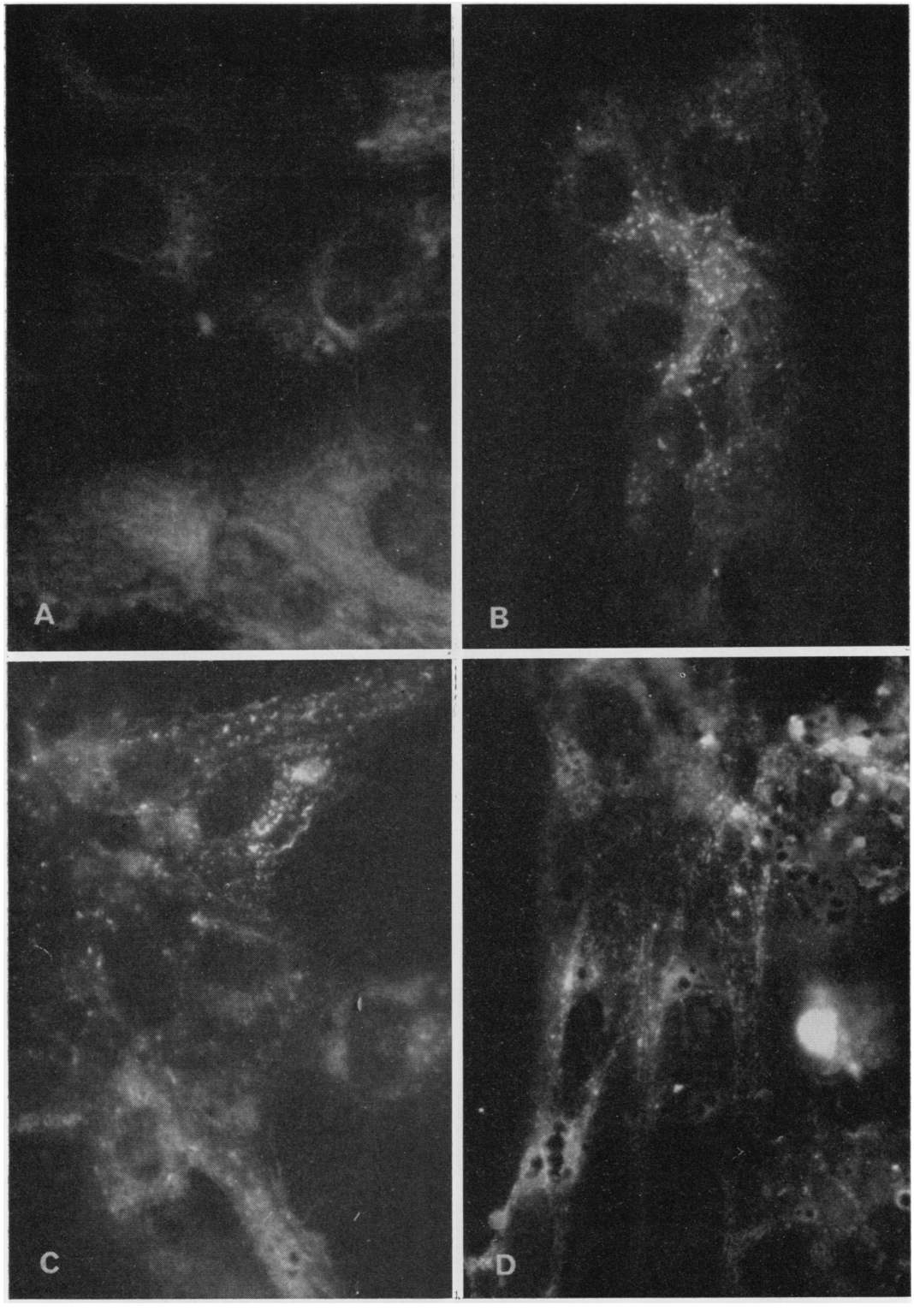

FIG. 2. *Reaction of unabsorbed anti-BAI strain A fluorescein-labeled antibody from the rabbit with normal chick embryo celis (A) and chick embryo cells (B) 2 hr, (C) 12 hr, and (D) 48 hr after exposure to strain MC29 virus.* ×675.

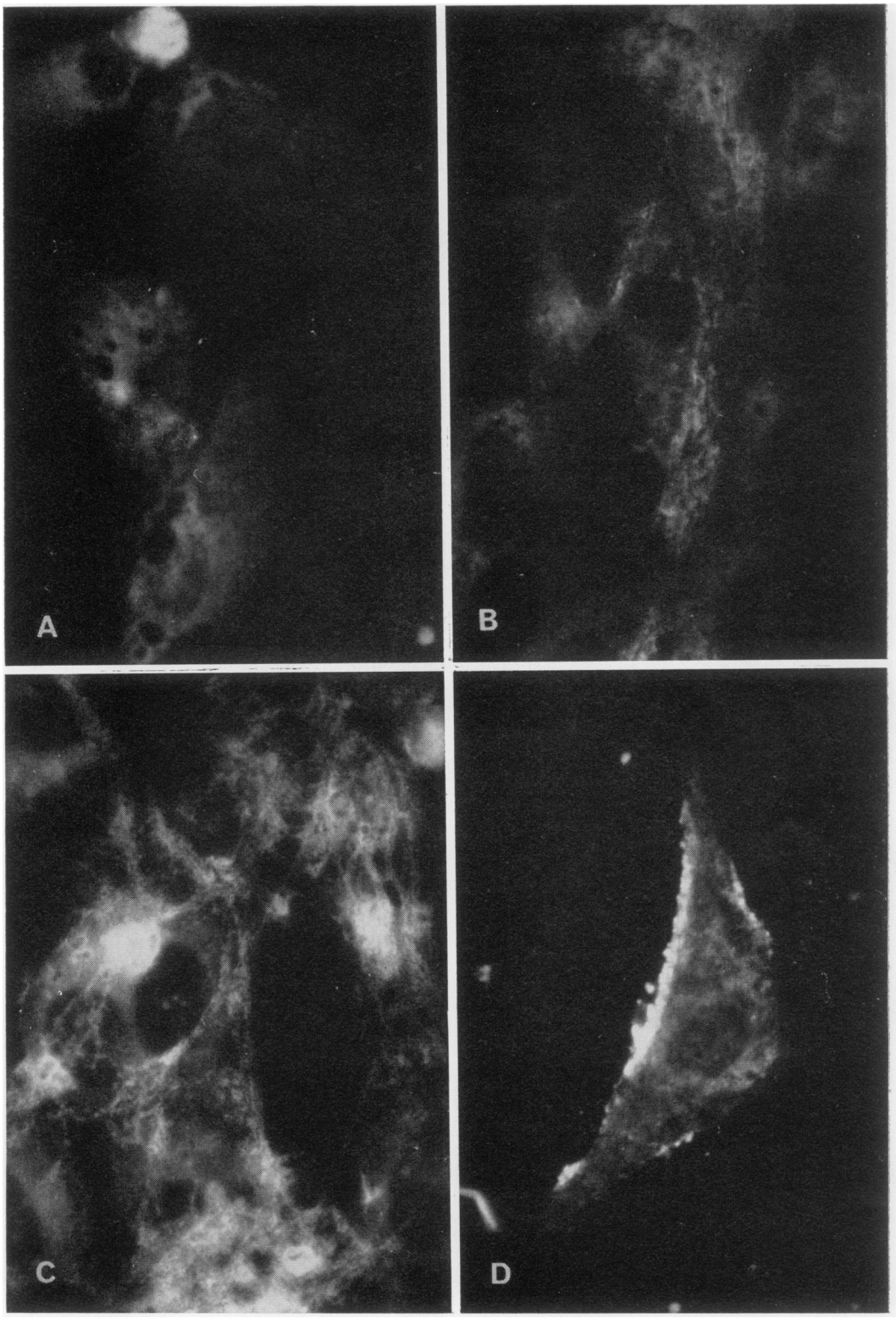

FIG. 3. *Reaction of absorbed anti-BAI A fluorescein-labeled antibody with chick embryo cells (A) 14 hr, (B) 36 hr, (C) 48 hr, and (D) 72 hr after exposure to strain MC29 virus.* ×675.

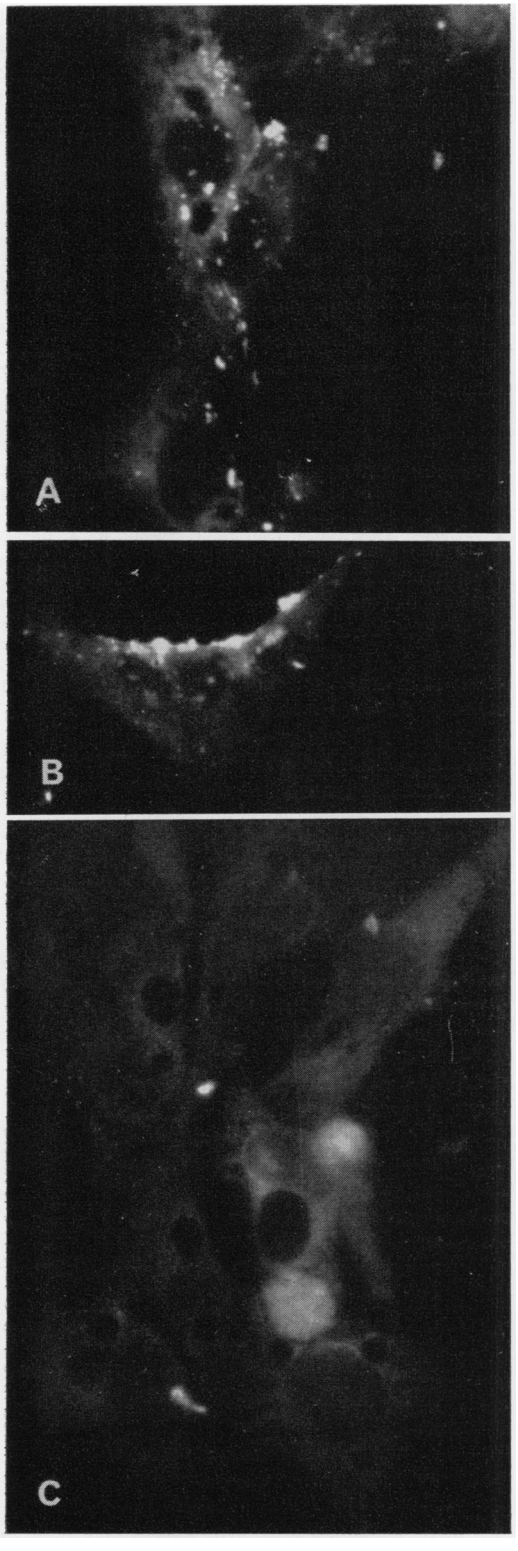

FIG. 4.

TABLE 1. *Focus-forming units (FFU) of MC29 virus liberated in chick embryo cell cultures infected with the agent*

Hr after infection	Hr fluid in contact with cells	FFU/ml ($\times 10^3$)
2	2	3.1
4	4	1.1
6	6	1.1
8	8	0.8
10	10	0.5
12	12	0.7
14	14	5.1
16	16	14
18	18	40
20	20	47
24	24	49
36	36	340
48	48	1,500
60[a]	12	540
72[a]	12	280

[a] The cells were trypsinized and passed 48 hr after infection; culture fluid was changed again at 60 hr.

BAI strain A agent occurs, however, only by immunization with the virus after suitable degradation of or damage to the particle envelope. Virus treated as with Tween 80-ether (6) produces antibodies in rabbits fixing complement with both coat and GS antigens. Immune serum produced in the rabbit with BAI strain A virus in the present work likewise contained antibodies against both coat and GS antigens (Fig. 1A and 1B) because of potassium tartrate damage (3) to the particles and consequent exposure of the internal antigen. Such immune serum should thus be capable of revealing the multiplicity of virus antigens within the infected cells as compared with the limitations of serum either from the chicken or hamster.

Despite the evident potential activity of the immune serum, it was surprising to observe the striking difference between the results with unabsorbed rabbit serum conjugate and those obtained with chicken and hamster immune sera by others (13, 20, 25, 27). This was marked by the granular fluorescence appearing regularly in the cytoplasm of some cells within 2 hr after exposure to virus and in increasing numbers of cells in the following 22 hr. The staining was not seen in normal control cells treated with unabsorbed serum, and it could be abolished by absorption with a large excess of normal cells. Whereas the labeling

FIG. 4. *Fluorescent antibody staining of chick embryo cells morphologically altered by strain MC29 virus at (A) 13, (B) 35, and (C) 56 days after exposure to the virus.* $\times$ *675*

thus appeared to be related specifically to cell response to infection with MC29 virus, the nature and origin of the stained particles were not clear. It seemed possible that they were cell components normally of low concentration but stimulated to rapid elaboration or increased concentration by cell interaction with virus containing antigenically similar constituents (7). Thus the material might be the result of initial stages of cell activity in the synthesis of components later assembled in the virus particles. It is notable that neither immune serum from the chicken (26) nor anti-GS serum (20) revealed such material in the early period after cell exposure to virus. That the staining was due to phagocytized virus or virus antigen seemed unlikely. Only a few cells stained at the beginning, and these contained many fluorescing particles. In view of the very large number of virus particles introduced, more than 10^3/cell, it might be expected that smaller numbers of particles might have been distributed in more cells. Furthermore, absorbed serum should have stained phagocytized virus antigen; instead, absorption of the serum with chick cells eliminated the early reaction.

Results obtained with the CEC-absorbed rabbit immune serum were similar to those found with other avian tumor viruses studied with chicken and hamster immune sera. Staining appeared only about 14 hr after exposure of the cells to virus, and was pronounced at 72 hr, a behavior closely related to the onset and rapid increase in virus output as seen in Table 1. As observed with other avian tumor agents, virus antigen appeared to be localized predominantly at the cell surface, which was in keeping with knowledge of the site of liberation of the agent (10). Diffuse cytoplasmic fluorescence was seen only in the very early stages of infection (14 to 24 hr after infection) and in very old cells (56 days after infection) of greatly decreased growth rate.

A particularly notable feature of the results was the progressive increase in the proportion of cells staining between about 14 and 72 hr. Under ideal conditions of contact, the inocula contained virus particles in numbers (about 1 FFU/cell or 1,000 virus particles/cell) sufficient to enter or infect all cells. Although antigen appeared in a few cells simultaneously with demonstrable virus liberation, response of all cells was delayed for approximately 60 hr longer. At this time all cells were altered in morphology and, consequently, must have been infected well before then. This behavior may well have been related to variations in the virus antigen-producing potentials (antigen concentration) of individual cells. In such case, response or lack of response to labeled antibodies provided no criterion of infection of CEC with strain MC29. It is of interest that the time sequence of staining of the cells infected with MC29 was essentially identical with that of CEC exposed to Rous sarcoma virus (26).

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