Detection of Adenovirus Type 12 Neoantigen(s) in a Continuous Human Amnion Cell Line (FL) by Immunofluorescence.* (30668)

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Huebner *et al*(1) described virus-specific complement-fixing (CF) antigens in extracts of tumors produced by adenovirus type 12. The same CF antigens have also been found in newborn hamster kidney cells transformed *in vitro* with adenovirus type 12 in this laboratory(2). Similar specific antigens have been found in SV_{40}-transformed human cells(3,4). The antigens have also been demonstrated by means of fluorescent antibody (FA) technics with the use of antisera from hamsters carrying tumors induced by the above 2 viruses (5-7). Furthermore, the antigens have been found in cultured normal cells infected with these viruses(8-10), and the term "neoantigens" was proposed by Huebner (personal communication) to designate these newly discovered antigens.

In view of the importance of neoantigens, both in virus infection and in transformation, we have studied by means of the FA technic the appearance of neoantigens in human amnion FL cells infected with adenovirus type 12. The results are reported in this paper.

Materials and methods. Cell lines. A clonal line (WT4-1) of human amnion FL cells, resistant to destruction by polioviruses, was used for most of the work reported here. A cloned subline (KB-34) of the KB cell was used for the preparation of virus stocks. All cell lines were carried in 32-ounce prescription bottles in Medium 199 with 10% fetal bovine serum. Medium 199 containing 2.5% fetal bovine serum with 0.4 mM additional arginine(11) was used for maintenance of infected cultures.

Virus strains. The prototype (Huie) strain of adenovirus type 12 was plaque-purified 3 times on human embryonic kidney cell cultures and designated pl-1041. The virus stock was prepared from KB cell monolayers infected at high input multiplicity and overlaid with the maintenance medium. The inoculated cultures were incubated at 37°C until cytopathic effects were complete (4 to 5 days) at which time the cells were harvested and centrifuged at 2,000 rpm for 10 minutes. The sedimented cells were resuspended in the supernatant fluid at 1/20th of the original volume, sonicated in a Bronwill Biosonik apparatus at 20 kc for 5 minutes, and centrifuged at 2,500 rpm for 10 minutes to remove cellular debris. The supernatant fluid was distributed into rubber-stoppered glass tubes in 5 ml amounts and stored at −20°C.

Similarly, adenoviruses type 7 (Pinckney strain) and type 18 (D. C. strain) were 3 times plaque-purified on human embryo kidney cell plates and respective virus stocks were prepared in KB cells. Adenovirus type 5 (Ad 75) was 3 times plaque-purified on human amnion FL cell plates. Virus stock was grown in KB cells.

Infection of coverslip cultures. Plastic petri dishes (60 × 15 mm, Falcon) were used. Four rectangular coverslips (7 × 22 mm) were placed into each of a series of dishes, and each dish was then seeded with approximately 5×10^5 cells contained in 5 ml of growth medium. The dishes were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air, and on the following day the growth medium was removed and 0.5 ml of virus, diluted to give a virus:cell multiplicity of 50:1, was distributed into each dish. After 2 hours' incubation at 37°C in a CO_2 atmosphere to allow for viral adsorption, 5 ml of maintenance medium was added per dish, and the plates were incubated for additional periods as indicated under *Results*.

Plaque titration of virus. Confluent monolayers of human embryonic kidney cells (third passage culture) in plastic petri dishes were inoculated with 0.3 ml of appropriate dilutions of the virus (adenovirus types 7, 12

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or 18) in Hanks' solution. A viral adsorption period of 2 hours' incubation in the humidified CO_2 incubator, with occasional gentle rocking of the plates, was used. The monolayers were then overlaid with 10 ml of Medium 199 containing 5% fetal bovine serum and 0.9% agar with 0.4 mM additional arginine. After 10 days of incubation, plates were reoverlaid with 5 ml of the same medium to which was added neutral red at a final concentration of 1:80,000. Plaque counts were made 2 to 3 days after staining. The same procedure, using FL cell plates, was employed for plaque assays of adenovirus type 5.

Viral antiserum. Adenovirus type 12 immune serum was prepared by injecting rabbits intravenously and subcutaneously at weekly intervals for a total of 10 courses. The virus stock consisted of plaque-purified, sonicated virus which was administered intravenously in 3 ml amounts and subcutaneously with 1 ml on each occasion. The animals were bled 2 weeks after the last injection.

Sera from tumor-bearing hamsters. Newborn hamsters were inoculated subcutaneously in the intrascapular region with 0.05 ml of undiluted stock adenovirus type 12, Huie strain. All animals were less than 24 hours old. Sera were collected as late as possible after the appearance of tumors. Complement-fixation tests were performed by our standard procedure adapted to use in the microtiter system(12) on pools of 5 to 6 sera. The pool used in these studies had a complement-fixing antibody titer of 1:16 against a CF antigen prepared as described by Huebner *et al*(1); the antigens were derived from cell lines established from hamster kidney cells transformed *in vitro*, and also from lines of cells established from tumors induced by adenovirus type 12.

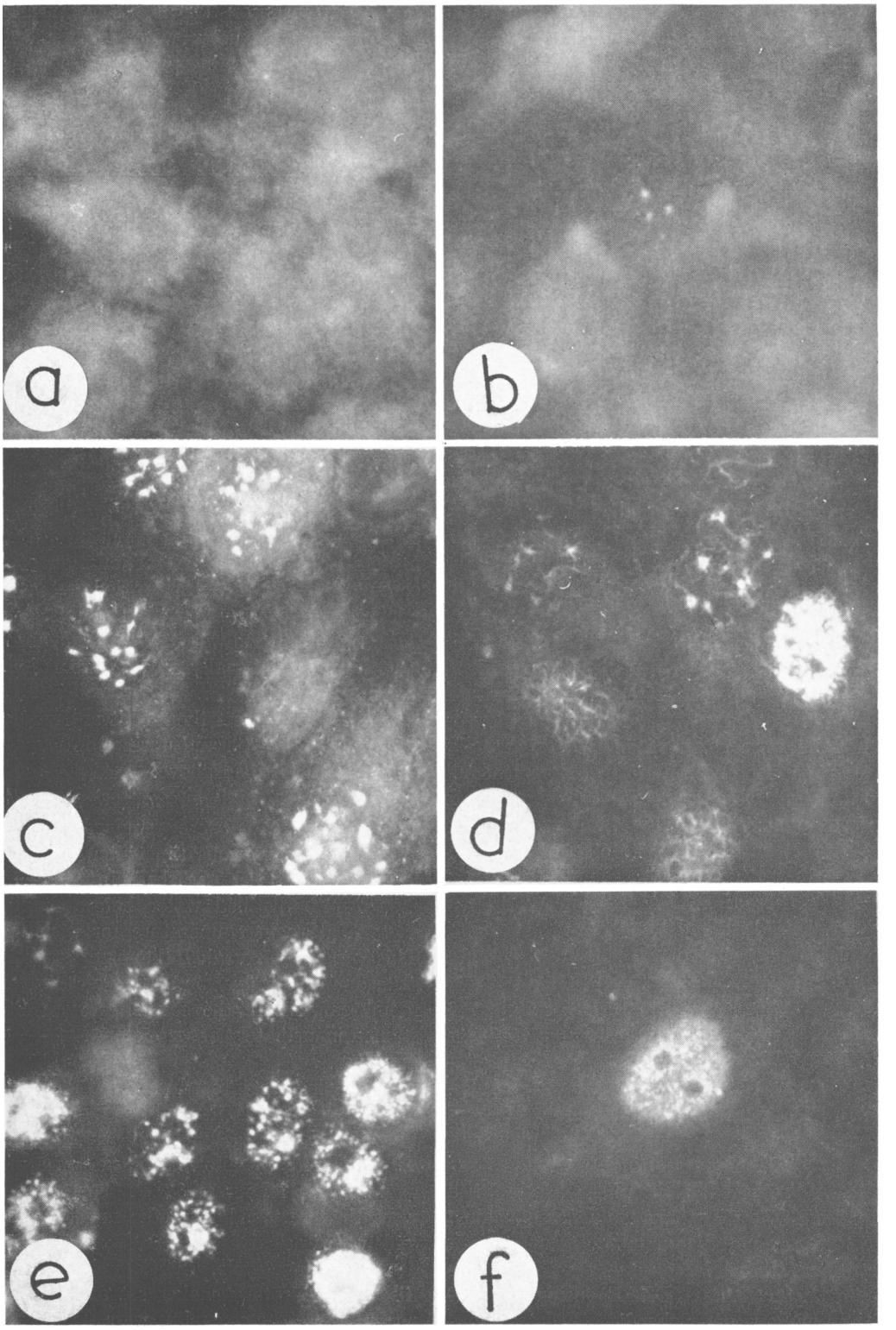
Antibody against hamster gamma globulin. To 50 ml of normal hamster serum was added an equal volume of saturated ammonium sulfate at 4°C. The resulting precipitate was collected, resuspended in phosphate buffered saline (PBS), pH 7.2, and dialyzed against PBS to remove the ammonium sulfate. It was then fractionated on a column of diethylaminoethyl cellulose (DEAE) using the procedure of Levy and Sober(13). Eluates showing maximum absorption at 280 mμ in a Gilford spectrophotometer were combined and reduced in volume by pervaporation. This preparation contained 0.28% protein. A goat was given 3 intramuscular injections of this preparation (2 ml per injection) in complete Freund's adjuvant at weekly intervals, and bled approximately 3 weeks after the last injection. Immuno-electrophoretic examination of the antiserum using whole hamster serum as the antigen showed 3 lines of precipitation, 1 a strong anti-gamma globulin line.

Conjugates. For the indirect test, the goat anti-hamster gamma globulin was precipitated with 50% saturation with ammonium sulfate, as previously described; the precipitate was dissolved in PBS and dialyzed to remove the excess ammonium sulfate. A protein determination was made using the biuret procedure and conjugation with fluorescein isothiocyanate was carried out using a dye to protein ratio of 1:100. Fractionation of the conjugate on DEAE was carried out as described previously(14). After dialysis with PBS, the conjugate was used without absorption procedures, usually at a dilution of 1:10 in PBS.

For the direct test the rabbit anti-viral serum was conjugated and purified in the same manner as described above.

Fixation and staining procedures. The 7 × 22 mm coverslips were removed from the petri dishes at various time intervals following infection, were washed in PBS, and dipped into distilled water before fixing in acetone at room temperature for 5 minutes. This procedure avoided the precipitation of salts during the fixation. After air drying at room temperature the coverslips were either stained immediately or were stored at −20°C until use.

In the indirect procedure the cell sheet on the coverslips was flooded with a 1:5 dilution in PBS of serum from tumor-bearing hamsters and was allowed to react in a moist chamber for 1 hour at 37°C. The coverslips were then removed from the moist chamber and were washed in PBS 3 times, 5 minutes each washing cycle, in Columbia staining dishes on a rotary shaker set at 130 cycles per minute. The coverslips were then drained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dry and stained with the labeled goat anti-hamster gamma globulin for 1 hour at 37°C in the moist chamber. Washing was effected as before, and the coverslips were then mounted in buffered glycerol (pH 7.2) and examined with the fluorescence microscope.

A similar staining and washing procedure was carried out in the direct test for viral antigen, utilizing only the labeled viral antibody.

Controls. The controls for the immunofluorescent test consisted of the following: (1) the staining of uninfected cells in the same manner as the infected cells; (2) the use of cells infected with adenovirus type 5 in the staining procedure; (3) the omission of the intermediate serum reaction in the staining procedure on infected cells; (4) the use of normal hamster serum as the intermediate serum in the staining reaction, and (5) the use, as the intermediate serum, of serum derived from hamsters bearing tumors induced by SV_{40}.

In addition to the above, cells infected with adenovirus type 18 or type 7 were removed from the cultures at 24 hours, fixed and reacted with serum from hamsters bearing tumors induced by adenovirus type 12, and stained as in the test procedure, to determine whether a cross reactivity exists between oncogenic strains of adenoviruses.

Growth curve. Cell monolayers of 1×10^6 cells in tubes were inoculated with 0.1 ml of stock virus at a virus:cell ratio of 50 PFU per cell. After 2 hours for virus adsorption at 37°C the cell sheets were washed 3 times with maintenance medium and 2 ml of new medium was added. At various times after incubation at 37°C the infected cells from two tubes were scraped into the medium, frozen and thawed 6 times, and centrifuged at 2,500 rpm for 10 minutes. The supernatant fluids were used for virus assay by the plaque technic on human embryonic kidney cell plates.

Results. Time of appearance of neoantigen(s). Coverslips of infected cells were removed and fixed at hourly intervals. The first appearance of the antigen was at 6 hours (Fig. 1, b), when only a very few cells showed the exclusively intranuclear pinpoint staining of the new antigen. The number of cells producing the antigen increased until approximately 12 hours post infection, when the majority showed the presence of the antigen (Fig. 1, c). The intensity of staining increased and the morphology changed with time.

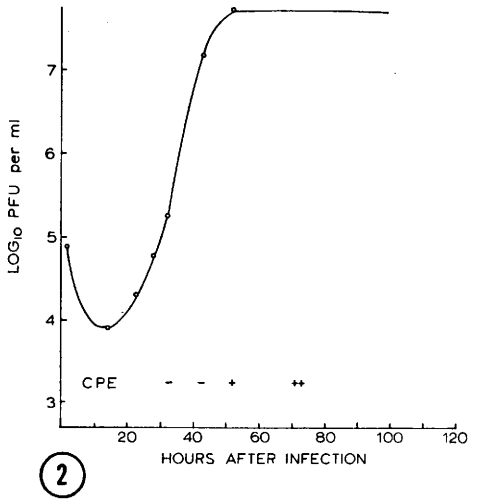
Morphology of the antigen. The first appearance of the neoantigen at 6 hours showed that the antigen formed at certain sites within the nuclei of the infected cells (Fig. 1, b). With time, the staining material extended out from these pinpoint sites until a more or less stellate configuration was evident (Fig. 1, c). The extension of the staining with time gives the impression that the antigen becomes filamentous throughout the nucleus (Fig. 1, d), and finally at later time periods it appears as fairly large granular forms within the nucleus (Fig. 1, e).

Growth curve. Newly formed infectious virus first appeared at approximately 24 hours after infection, as can be seen from the growth experiment illustrated in Fig. 2. The amount of virus recovered then increased until about 48 hours post-infection when viral increase plateaued and remained relatively constant until 120 hours post-infection, the last interval at which assay was done. The maximum titer was 5.7×10^7 PFU/ml representing a virus yield of approximately 114 PFU/cell.

Beginning cytopathic effects were first observed at 52 hours post-infection. Cytopathogenesis increased with time, with 4+ degeneration evident by 120 hours.

Controls. No staining was seen in the control preparations. However, it was found that when the sera from hamsters bearing tumors induced by adenovirus type 12 were reacted with cultures infected with other oncogenic

FIG. 1. Human amnion cells (FL) infected with adenovirus type 12 stained with labeled antibody for neoantigen and viral antigen (original magnification ×500). (a) Fixed and stained by the indirect procedure for neoantigen 4 hr post-infection. (b) Same procedure, 6 hr post-infection. (c) Same procedure, 12 hr post-infection. (d) Same procedure, 30 hr post-infection. (e) Same procedure, 44 hr post-infection. (f) Fixed and stained by the direct procedure for viral antigens, 30 hr post-infection.



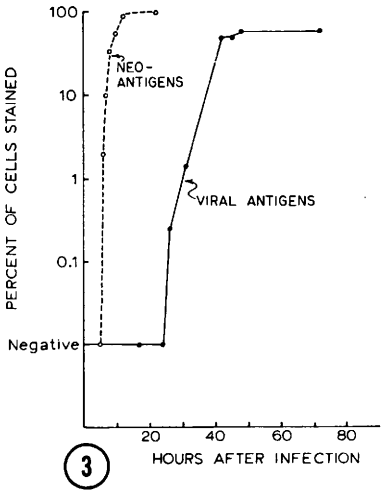


FIG. 2. Growth curve of adenovirus type 12 in a clonal line of human amnion FL cells.

FIG. 3. Development of neoantigens and viral antigens in a clonal line of human amnion (FL) cells infected with adenovirus type 12 (pl-1041) and stained with fluorescein conjugated antibody (see text).

adenoviruses (adenovirus types 7 and 18) and the cells then treated with the labeled anti-hamster gamma globulin, staining resulted with approximately the same morphological characteristics as when the adenovirus type 12 infected cultures were tested.

Time of appearance and morphology of viral antigen. Direct staining of infected cells with labeled rabbit serum containing antibody to the virion gave an entirely different picture from that of the indirect procedure which identified the neoantigen(s), *i.e.*, the use of labeled serum from hamsters bearing tumors. No staining was evident until approximately 24 hours, at which time viral antigen was detectable in the nucleus of only a few scattered cells. The staining pattern of the viral antigen differed notably from that of the neoantigen, *viz.*, early in the infectious process the entire nucleus with the exception of the nucleoli (Fig. 1, f) stained brightly. The number of cells showing staining increased with time (Fig. 3) and staining then extended into the cytoplasm. After further incubation, the cytopathic effect of the virus was evident from the appearance of the brightly stained round cells.

Discussion. The exclusively intranuclear formation of the neoantigen described in this study differs from that reported by Pope and Rowe(6), who noted both intranuclear and intracytoplasmic staining "flecks" of antigen in cultures of hamster and human cells infected with adenovirus type 12. The intermediate serum used in the staining procedure described in the present study was not tested for its complement-fixing ability with any other antigens either viral or virus-derived, *e.g.*, the "C" or "A" antigens reported by Klemperer and Pereira(15). The exact identity of the antigen(s) formed in the FL cell system was therefore not determined beyond the fact that it reacted with serum derived from hamsters bearing tumors induced by adenovirus type 12. The antibody was not type-specific, since it reacted with material in cells infected with either of two other oncogenic adenovirus types (7 and 18); it did not, however, react with cells infected with adenovirus type 5 (at present not known to be oncogenic). Likewise, serum obtained from hamsters bearing tumors induced by SV_{40} failed to react with the neoantigen of adenovirus type 12.

The temporal difference in the appearance of the neoantigen and of the viral antigen is one of the salient findings of this study. The neoantigen was first detected at 6 hours, and the number of cells in which it was found increased until the majority showed its presence at 12 hours post-infection. This time period correlated closely with the lowest in-

flection of the viral growth curve and preceded by about 12 hours any detectable viral antigen stainable with fluorescein-labeled antibody to the virion.

Neoantigens may have a role in the formation of viral precursor substances induced by infection with the virus. Up to this point, however, we have found that only the oncogenic adenoviruses among the types tested possess cross-reacting antigens, although it seems probable that neoantigens are to be found in every viral infection. An example of early proteins being formed during viral infection was described for bacteriophage by Wiberg *et al*(16). More recently, Hamada and Kaplan(17), in the case of animal viruses, showed by means of an indirect precipitation test that early during the infection of rabbit kidney cells with pseudorabies virus, specific proteins appeared which bear no precursor relationship to the viral particles (nonstructural viral proteins). These proteins, however, were antigenically distinct from those found in noninfected cells. The stainable antigen described in the present report may or may not be concerned with the oncogenic potential of the virus, hence there is some justification to use Huebner's designation of "neo-antigen" until additional information on their nature accrues.

It would be of importance to know how many of the adenovirus immunotypes currently not known to be oncogenic possess neoantigens serologically related to those of the oncogenic immunotypes. The sharing of such cross-reacting neoantigens may serve to reveal oncogenic potential, and a study on this aspect is currently under way in this laboratory.

The relative ease of preparing the test system, the means to differentiate the newly-induced neoantigen from the viral antigen, and the distinctive morphology of the staining pattern of the new antigen provide an approach to determining whether adenoviruses are involved in the causation of cancer in man.

Summary. Neoantigens produced in FL cells by infection with oncogenic types of adenoviruses were stained by the indirect FA technic using serum from hamsters bearing tumors induced by adenovirus type 12 as the intermediate serum. The neoantigen of adenovirus type 12 first appeared at 6 hours post-infection, and thereafter the amount within the cell (nucleus) increased until 12 hours. The number of cells in which the antigen was demonstrable also increased until at 12 hours post-infection the majority of the cells contained the antigen. Formation of neoantigen preceded the formation of new infectious virus, as was shown by growth curve experiments and by staining with fluorescein-labeled antibody against the virus.

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