The Pathogenesis of Yaba Virus-induced Histiocytomas in Primates*

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SUMMARY

Benign histiocytomas, detectable histologically by the 3d day, regularly followed subcutaneous inoculation of cell-free saline suspensions of the tumor into rhesus monkeys. Proliferation proceeded rapidly during the initial 2 weeks, producing a tumor of characteristic cell type and pattern. No cell other than certain primate tissue histiocytes has been shown to be a susceptible host. Regression was an individual cell phenomenon, beginning while proliferation was still active but gaining in prominence over a period of 2-3 months. Circulating neutralizing antibody was ineffective in preventing growth of the established tumor. Following tumor regression, plasma cells and lymphocytes marked the site, but fibrosis did not occur.

Intravenous inoculation of virus was followed by the appearance of many tumors in heart, lungs, muscles, and subcutaneous tissues of the monkeys. Similar tumors developed locally following direct inoculation of virus incorporated in Freund's complete adjuvant into liver, spleen, or kidney.

No tumors developed when virus was injected subcutaneously into rats, guinea pigs, and rabbits. Although histiocytes predominated in the guinea pig reaction, the cells were not transformed, and fibrosis developed at the inoculum site.

Yaba virus infection in susceptible monkeys provides an interesting experimental model because of the magnitude and rapidity of the proliferative response. Tumors produced by this virus were first reported by Bearcroft and Jamieson in 1958 (2) following an outbreak of subcutaneous masses in their monkey colony in Yaba, Nigeria. The outbreak involved twenty Asiatic monkeys which were housed in open-air pens. Andrews and co-workers (1) identified the etiologic agent as a virus resembling members of the pox virus group.

Niven et al. (10) studied a number of well-established growths by electron and light microscopy and concluded that the subcutaneous masses consisted of altered fibroblasts containing virus particles in the cytoplasm. The virus was thought to be of the DNA type based on histochemical observations according to methyl-green pyronine, acridine orange, and Feulgen reactions. Electron microscopy revealed brick-shaped particles not unlike Shope fibroma and molluscum contagiosum viruses. They noted that regression of the masses regularly occurred within 4-5 weeks and that the animals were subsequently immune to re-infection.

In the four tumors studied histologically by Niven et al. extensive necrosis was described in one and plasma-cell histiocytic response was observed in the regression phase of all.

In the course of detailed studies on the development and regression of the monkey lesions in our laboratory, we noted several features which were somewhat at variance with previous reports. These include the type of cell involved in the proliferative response, the neoplastic nature of the lesions and the characteristics of regression.

MATERIALS AND METHODS

A 22-day-old monkey tumor was minced in cold 0.14 M NaCl to effect a 25 per cent w/v suspension. Mechanical homogenization (Omni-mixer) was performed with the container immersed in an ice bath. The homogenate was centrifuged at 4,000 r.p.m. in an International PR-2 for 20 minutes at 4° C., and the supernate was passed through an HA (0.45 µ) Millipore filter. The filtrate was inoculated intradermally into two rhesus monkeys at multiple abdominal sites. The inocu-
lum for each site contained approximately 100 I.D.\textsubscript{50} of virus as measured by cutaneous titration in the monkey.

One injection site from each monkey was excised 24 hours later under local anesthesia. Sites were subsequently excised daily for the first 2 weeks, on alternate days during the 3d week, and at longer irregular intervals thereafter. Another group of monkeys similarly given inoculations was used for studies of older, regressing phases of the disease. The specimens were fixed in Lillie's modification of Carnoy's solution containing alcohol, formalin, and acetic acid. In addition, cryostat sections and fresh imprints were prepared. Histologic stains included hematoxylin and eosin, Papanicolaou, Wilder's silver impregnation for reticulin, Masson's trichrome connective tissue stain, periodic acid-Schiff (PAS) reaction for glycoprotein, Sudan red for neutral fat, and Nile blue sulfate for phospholipide. Acridine orange, methyl green pyronine, and Feulgen reactions were utilized for nucleic acid studies.

In other studies, the virus was inoculated intravenously into one group of rhesus monkeys; and in another series, the virus was incorporated in Freund's complete adjuvant and injected directly into various organs. Complete necropsies were performed on all these animals, and all organs, including the central nervous system and bone marrow, were sectioned.

To investigate the host range more fully, young adult rats, guinea pigs and rabbits were given injections intracutaneously of 100 monkey I.D.\textsubscript{50} of virus at multiple sites on the abdomen. Injection sites were later excised under local anesthesia at specified intervals and processed as the monkey tumors.

**RESULTS**

Injection of the virus into cutaneous and subcutaneous tissues of the monkey produced minimal signs of local trauma. No areas of necrosis were observed, and the neutrophil reaction to needle injury persisted only 1 day. At 48 hours post-inoculation, histiocytes predominated, and these were distributed along the needle tract and deep fascial plane. By the 3d day, the majority of histiocytes in the injection area showed specific alterations, and mitotic activity was evident. The nuclei of these cells were 2—3 times normal size and were oval or curved. Chromatin was coarse, irregularly distributed, and usually margined near a thickened nuclear membrane. The nucleoli were markedly enlarged and seemed more numerous.

On the 4th and 5th days following inoculation of the virus, the proliferating altered histiocytes formed compact 2- to 3-mm. nodules which were usually palpable (Fig. 1). Three to four mitoses were seen per high-power field. No necrosis or tuberculoid lesions developed, and the epidermis remained unaltered.

During the 2d week proliferation predominated, and a moderately circumscribed but infiltrative tumor was formed in the dermis and subcutaneous tissues. The proliferating cells became elongate with spindle-shaped nuclei and relatively scanty cytoplasm closely resembling fibroblasts. The tumor assumed a definite architecture, with parallel bundles of cells intertwined among capillaries in a storiform or woven-mat pattern (Fig. 2). The fusiform cells did not parallel capillaries as in granulation tissue, or form nodules of a granulomatous character with necrosis. This pattern was best seen from the 9th to the 13th day when the tumor measured 5—10 mm. in diameter.

A second feature which could be observed in some cells as early as the 8d day was the appearance of cytoplasmic inclusion bodies. However, this feature was relatively inconspicuous during the phase of most active cell proliferation. The inclusions became progressively more apparent during the 8d week and thereafter. These usually appeared first as dense, round, sharply defined, paranuclear basophilic structures. The cytoplasm at this stage seemed swollen and pale. The smaller bodies frequently grew to eventually occupy a large portion of the cytoplasm with eccentric displacement of the nucleus (Fig. 3). The larger masses, developing later, had vague outlines, irregular contours and were often amphophilic. Both types of bodies were periodic acid-Schiff-negative. Irregular cytoplasmic granularity was usually seen early, with later development of increasing numbers of vacuoles and globules of neutral fat, at which time the cells became swollen and rounded. Staining for phospholipide was negative. The cytoplasmic masses showed the red fluorescence of RNA with acridine orange and were pyronine-positive. Green fluorescent bodies corresponding to methyl green staining granules were smaller and less numerous. The characteristic RNA reactions of the nucleolus served as a good technical control.

After the 3d week, proliferative activity declined, and the large vacuolated, nondividing cells became more numerous. However, throughout the course of the disease, both types of cells could be found. Often spindle cells in mitosis were observed adjacent to the larger degenerating cells with pyknotic nuclei. The tumors attained a size of 2—5 cm. before regressing. The overlying epidermis frequently ulcerated and showed bacterial invasion.
However, ulceration was not a prerequisite for regression, since some lesions regressed with the overlying epithelium still intact.

Degeneration of the tumor appeared to be an individual cell phenomenon, and no zones of necrosis appeared, except in relation to ulceration (Fig. 4). Multinucleate forms occurred. These seemed to be an end stage of incompletely divided cells which contained many large dense inclusions. Following cell disruption numerous amphophilic masses could be seen in extracellular locations and occasionally in blood vessels.

The reticulum pattern was not greatly affected by the development of the tumor, and at no time was it destroyed (Fig. 5). Tumor cells proliferated within the original framework of the subcutaneous tissues and dermis, separating larger fibers as they became swollen. Delicate fibrils remained or perhaps developed between the tumor cells but were never conspicuous. Collagen was not laid down in the tumor even during regression, although a few normal fibroblasts penetrated from the periphery. Scar formation was not a feature of healing. Regression appeared to be a simple process of tumor cell disintegration.

Host cellular reaction to the tumor was apparent as early as the 5th day, became more intense during the following week, but remained at the periphery of the tumor until regression was well advanced. The reaction was characterized by perivascular collars of plasma cells, lymphocytes, histiocytes, and a few eosinophilic leukocytes (Fig. 6). Terminally, these cells predominated as the tumor cells dwindled and disappeared, and they remained at the site when no intact tumor cells could be found, although vague cytoplasmic masses were long in evidence. The reactive histiocytes and a few multinuclear giant cells of the foreign body type engulfed cell remnants as the tumor regressed, but these were not altered by the phagocytized material.

Both monkeys receiving the multiple inocula developed colitis and were sacrificed in the 3d week. Other animals were used for the study of older tumors. The internal organs of the animals receiving intracutaneous injections of virus showed no evidence of metastases or other viral effects. The colitis appeared histologically nonspecific.

Three monkeys were given inoculations intravenously of 1.0 ml. 100,000 I.D.₅₀. These animals lived 8–4 weeks. Terminally, they all had diarrhea. Two were sacrificed when moribund; the other was found dead. The findings at autopsy were similar in all three. The subcutaneous tissues throughout the body, but most prominently on the extremities, were studded with tumor nodules similar to those developing at the site of cutaneous inoculation of the virus (Fig. 8). Hundreds of tumors also permeated the muscles. Grossly, these were discrete or partially confluent, but microscopically tumor cells were seen extending among muscle fibers (Fig. 9). Many 1- to 2-mm. nodules were found in the subepicardial tissues of the heart, primarily in the right atrium and ventricle (Fig. 10). The lungs were dotted with subpleural nodules. A few tumors were found in the deeper parenchymal areas. Lymph nodes were enlarged, discrete, and soft. The spleens were enlarged but had good preservation of architectural features. The colon was inflamed but not ulcerated. The livers were mildly fatty; no tumors were noted.

The tumors in muscle, subcutaneous tissue, heart, and lungs were composed of cells similar in all respects to those of the 2- to 3-week-old tumors following cutaneous virus inoculation. In the lungs, neutrophils were more numerous and were presumably related to superimposed bacterial infection. The lymph nodes and spleen were hyperplastic with an increase in reticuloendothelial and plasma cells, but no tumor cells were found.

The diarrhea was probably unrelated to the virus infection, since no specific changes were noted in the bowel and since diarrhea occasionally occurred in uninoculated animals. However, the severe diarrhea may have played a role in the terminal illness, since extensive vacuolization of the renal tubular epithelium suggested a severe hypokalemia. Cytoplasmic inclusions or nuclear alterations were not observed in any cells other than in those of tumor nodules. The brain, salivary glands, and reproductive organs appeared entirely normal. The degree of fatty metamorphosis in the liver was commensurate with the diarrhea.

The intravenous inoculation experiments were later repeated with another group of monkeys. These animals showed the same wide-spread distribution of tumors, but they did not develop diarrhea, and three out of four survived. The subcutaneous and muscle tumors of the surviving animals eventually regressed.

Direct organ inoculation of virus incorporated in complete Freund’s adjuvant produced tumor nodules in the injected organs (liver, spleen, and kidney). Virus without adjuvant did not. The tumor cells were identical to those previously described. However, these tumors were accompanied by more lymphocyte, foreign body-type giant-cell and plasma-cell reaction, and contained foci of necrosis. This variation was attributed to reaction to the adjuvant. There was evidence of some permeation of tumor into the liver, following the pattern of the portal areas. This created cordlike ex-
tensions radiating from the site of major tumor growth. Older nodules became heavily fibrosed and calcified. This response was not seen without adjuvant.

Further support for the histiocyte as the cell of origin of the tumors was obtained by simultaneous inoculation of virus and an India ink suspension. Histiocytes which phagocytised large amounts of ink did not divide and eventually disappeared. However, some cells containing both virus and ink proliferated to form typical tumors. India ink particles could be seen in the cytoplasm of tumor cells containing characteristic virus inclusion bodies and altered nuclei (Fig. 7).

The antibody response of animals receiving subcutaneous inoculations of virus was also studied. The methods were previously reported (8). Chart 1 represents the serum complement-fixing antibody titers of monkeys at various times after inoculation. Antibody was detectable by the 4th-5th days, and the titers remained high until the animals died or were sacrificed.

No tumors resulted from subcutaneous inoculation of rats, rabbits, and guinea pigs with the virus. The immediate and persisting reaction in the rat was largely polymorphonuclear (Fig. 11). However, by the 10th day, moderate numbers of lymphocytes and histiocytes were observed with intact. Tumor cells were round, finely vacuolated, and many had large, pale intracytoplasmic inclusions. Others were in mitosis. H. & E. stain, ×400.

Fig. 2.—Ninth postinoculation day. Spindle-cell forms were arranged in a storiform pattern. H. & E. stain, ×400.

Fig. 3.—Twenty-second day. The epidermis was thin, but the neutrophils. In the rabbit, necrosis was more striking (Fig. 12). Nuclear fragmentation and scar tissue formation were evident by the 4th day.

The guinea pig showed more histiocytes among the neutrophils by the 4th day post-inoculation. These cells never became altered, as in the monkey, and cytoplasmic inclusion bodies were not seen. The site of injection was thickened by new fibroblasts and capillaries in 10 days (Fig. 13). These cells were oriented along the original tissue planes, as in the ordinary healing process, and showed no tendency to form a cohesive mass with distinctive architecture, as observed in the monkey.

DISCUSSION

These studies indicate that the histiocyte is the cell giving rise to tumors in monkeys infected with Yaba virus. Tissue histiocytes migrate to the area of virus inoculation, undergo striking morphologic alterations in a short period of time, and rapidly proliferate to form masses of neoplastic nature. The tumors are composed of aggregates of altered histiocytes and show specific architectural features with good preservation of the reticulum structure. The altered histiocytes closely resemble fibroblasts during their active proliferative stage and could easily lead to erroneous conclusions regarding their origin, unless closely spaced sequential histologic studies were done. This is not surprising, since histiocytes are recognized as facultative fibroblasts, and they may assume this form when actively proliferating or stimulated to tumor production (7).

The storiform pattern (from Storia-mat) which is best demonstrated in the Yaba tumors during their early rapid growth phase, closely resembles the histologic appearance of human fibrous xanthoma. This human lesion develops from histiocytes, accumulates varying amounts of stainable lipide and is usually benign, although malignant human histiocytomas are recognized.

Unlike the usual fibroma, the spindle cells in the Yaba histiocytoma are not accompanied by collagen fiber elaboration, and the tumor never appears fibrotic. In this regard, it differs from the virus-induced rabbit and deer fibromas of Shope (11, 12).
FIG. 5.—Reticulum was intact within the area of tumor proliferation. Wilder stain, X400.

FIG. 6.—Perivascular plasma-cell reaction confined to the periphery of the histiocytoma during active proliferation and early degeneration. H. & E. stain, X400.

FIG. 7.—Yaba virus monkey histiocytoma, 23 days following simultaneous inoculation of virus and India ink. Tumor cells with altered nuclei had black ink particles and lighter inclusion bodies in the same cells. H. & E., X400.

FIG. 8.—Multiple subcutaneous nodules in extremities of a rhesus monkey 23 days following I.V. inoculation of Yaba virus.
Fig. 9.—Multiple tumors in skeletal muscle 23 days after I.V. inoculation of virus. H. & E., ×50.

Fig. 10.—Multiple tumors in subendocardium of right ventricle 23 days after I.V. inoculation of virus. H. & E., ×50.

Fig. 11.—Neutrophil reaction to subcutaneous inoculation of Yaba monkey histiocytoma virus into the rat. Second day. H. & E., ×400.

Fig. 12.—Neutrophil and mononuclear reaction with nuclear fragmentation after similar inoculation into the rabbit. Fourth day. H. & E., ×400.

Fig. 13.—Fibroblast formation with collagen deposition at the site of Yaba virus inoculation in the guinea pig. Tenth day. H. & E., ×400.
Although morphologically recognizable virus was seen by electron microscopy only in the cytoplasm of tumor cells by Niven et al. (10) and Owens, there is a prompt and permanent nuclear alteration with the acquisition of morphologic features of neoplasia. Cytoplasmic dense bodies or “inclusions” are a prominent characteristic of the Yaba cell. These probably do not represent virus alone, since they regularly fluoresce red and are pyroninophilic. Green fluorescence and methyl green-stained particles in smaller granular form in the cytoplasm may represent aggregates of DNA virus particles. Owens observed numerous virus particles distributed diffusely in the cytoplasm of the monkey tumor cells. However, in human tumors produced by this virus, the virus particles were arranged in cytoplasmic aggregates surrounded by double membranes. The well delineated juxtanuclear cytoplasmic masses which appear early and occur in the active spindle forms may represent some form of immature virus, since recognizable virus is rarely seen in these. The larger, vaguely outlined amorphophilic masses which predominate in cells of regressing tumors contain large numbers of typical mature virus particles intermingled with amorphous material which probably represents residual cytoplasmic debris. This could account for the tinctorial properties of the large bodies.

Two types of intracytoplasmic inclusions have been described in cells infected by other pox viruses. Bland and Robinow (8) reported that Giemsa-stained tissue culture cells infected with vaccinia showed small, round, purple cytoplasmic masses within a few hours after infection. Later, more diffuse masses appeared which had a Feulgen-negative network and Feulgen-positive particles. More recently, Higashi reported similar findings (6). Infection of HeLa cells with ectroinulia virus, eosinophilic masses resulted in the prompt appearance of small, circular or oval, basophilic, Feulgen-positive cytoplasmic inclusions surrounded by a clear zone. At a later stage these inclusions tended to be larger and more dispersed. In the case of ectroemia virus, eosinophilic masses also developed late and were Feulgen-negative. Regression of the Yaba tumor in the monkey may be in large part due to an in vivo cytopathic effect of the virus. Neutralizing and complement-fixing viral antibody appears rapidly in the serum of infected monkeys and is high during the phase of tumor growth (Fig. 18). However, previous studies showed that, although viral antibody was effective in preventing infection, it seemed to have little effect on the progression of established tumors (8). In addition, we found little histologic evidence that cellular immune mechanisms, as measured by plasma cell and lymphocyte responses, contributed significantly to tumor regression, since these cells remained peripheral to the tumor until cell disintegration was well advanced.

There is no indication, thus far, that these neoplasms are potentially malignant. The widespread tumors observed following intravenous virus inoculation were probably owing to viral induction in multiple sites rather than to true cellular metastases. Such a mechanism has been postulated in the development of secondary Rous sarcomas in the chicken lung. There is no evidence that the tumor cells themselves tend to migrate.

Only a narrow range of species susceptibility to the Yaba virus has been demonstrated. A number of nonprimate laboratory animals, including mice, rats, hamsters, rabbits, dogs, and cats, were found by Felts to be refractory to the virus (4). Human susceptibility to intentional or accidental inoculation has been reported (5). The tissue response was found to be similar to that of the monkey.

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