Studies on a Transplantable Chicken Tumor (RPL-12 Lymphoma)

II. Mechanism of Regression Following Infection with an Oncolytic Virus*

ROBERT LOVE AND GEORGE R. SHARPLESS

(Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.)

Regression or retardation of growth of transplantable tumors can be readily induced by infection with a number of viruses (3, 4, 5, 8, 14, 19). The possibility of utilizing viruses in the treatment of neoplastic disease has been limited, because, although the tumor regresses, the host usually succumbs to the virus infection. The ability of five neurotropic viruses to induce regression of the RPL-12 chicken lymphoma in the pectoral muscle has been demonstrated by Sharpless et al. (19). Although the viruses used (Russian spring-summer, West Nile, Japanese B and St. Louis encephalitis, and looping ill viruses) are highly pathogenic for many species, and despite the fact that virus multiplied in the host, the chickens showed no clinical signs attributable to virus infection. Subsequently, a more potent oncolytic strain (N.F.T.) of St. Louis encephalitis virus was isolated in this laboratory from the RPL-12 tumor (17).

Some of the possible mechanisms involved in the phenomenon of virus-induced oncolysis have been discussed in a previous paper (8), in which it was suggested that the ideal oncolytic virus is one which will multiply in, and destroy tumor tissue only. Viruses which have proved to be of value in the treatment of neoplastic disease in animals have a selective affinity for tumor tissue, i.e., they can be isolated in greater amounts from the tumor than from most other tissues (3–5, 8, 14, 19). The present work is a study of the mechanism of oncolysis and an attempt to assess the relative pathogenicity and infectivity of N.F.T. virus for the tumor and for the host and, in particular, to discover whether the presence of a tumor would increase or decrease the pathogenicity of the virus for the chicken.

When regression of a transplantable tumor is produced, two factors must be considered: the action of the oncolytic agent and the contribution of the host defenses. This is especially important, because it has been shown (9) that the transplantation of suitable amounts of the RPL-12 lymphoma into the pectoral muscle can produce a tumor which later regresses; after a period of progressive growth, tumor cells undergo phagocytosis, and the observation of plasma cell hyperplasia in the spleen and subsequent development of resistance to further transplantation strongly suggest that immune mechanisms are involved. In the present study we have utilized the observations of Shirai (20), Murphy and Sturm (15), and Greene (1), who have shown that tumors transplanted in the brain provoke less host reaction than in any other site of transplantation. By comparing the effects of virus infection on the RPL-12 lymphoma growing in the pectoral muscle and in the brain, we have attempted to evaluate the contribution of the host defenses in bringing about regression of the tumor.

MATERIALS AND METHODS

Birds, preparation of tumor inoculum and biological procedures.—Most of these procedures have already been described (9). In some experiments, however, the tumor suspension was inoculated intracerebrally (Table 1) by insertion of a 22-gauge needle through the parietal bone into the left cerebral hemisphere. Autopsies were performed, and tissue sections were prepared and stained as before (9), except that in Experiments III, IV, and V (Table 1) the following additional blocks of tissue were taken: coronal sections through the anterior cerebrum, optic chiasma, lower thalamus and optic tectum, cerebellum and pons, and medulla; transverse sections of the cervical, brachial, thoracic, and lumbar spinal cord. Paraffin sections of the central nervous system were also stained with buffered thionine of pH 5 (6) to observe the Nissl substance, and by the Loyez method for myelin (18). Lendrum's reticulin (7), Weigert's resorcin fuchsin (18), and McFarlane's...
pico-Mallory (18) stains were also used to evaluate the nature of vascular degeneration in a few sections. The periodic acid Schiff reaction (P.A.S.) of McManus (19) was found useful in demonstrating cytoplasmic inclusions.

**Virus inoculum.**—The virus was a strain (N.F.T.) of St. Louis encephalitis virus which was isolated in our laboratory in the course of studies with another virus (17). The virus was propagated in tumor-bearing birds by intramuscular injection at a site distant from the tumor; the infected tumor was harvested 3 days later. A 10 per cent suspension of the infected tumor tissue was made in distilled water and preserved by desiccation from the frozen state. The virus inoculum for the various experiments was prepared by resuspending this dried material in water. Except in Experiment I, in which a 0.1 per cent suspension was used, the concentration of the virus inoculum was determined by giving mice intracerebral inoculations of serial tenfold dilutions of infected tissue in saline, four mice being used for each determination, and the time when birds were killed for pathological examination. Birds were always killed between 10 and 12 A.M. each day to avoid confusion between physiological (diurnal) and virus-induced alterations of the mitotic rate. In most experiments, virus-infected and uninfected birds were divided into two groups as before (9): the first group was killed as shown in the table, and the second was set aside for observation of tumor development, sickness, and mortality over a period of 3 months.

In view of the recent work of Kidd on the regression of transplanted lymphomas following the inoculation of normal guinea pig serum (2), an additional control experiment was run.

**TABLE 1**

**EXPERIMENTAL DATA AND RESULTS**

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>INOCULUM</th>
<th>BIRDS KILLED FOR PATHOLOGY STUDIES</th>
<th>BIRDS UNDER OBSERVATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PURPOSE</td>
<td>PER CENT TUMOR IN SUSPENSION AND ROUTE OF INOCULATION</td>
<td>DAYS AFTER TUMOR INOCULATION</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ROUTE OF INOCULATION</td>
<td>TOTAL NO. OF BIRDS</td>
</tr>
<tr>
<td>I</td>
<td>Virus infection on tumor growth in muscle</td>
<td>1.0 I.M.</td>
<td>None</td>
</tr>
<tr>
<td>II</td>
<td>Virus infection on tumor growth in muscle and in brain</td>
<td>1.0 I.C.</td>
<td>None</td>
</tr>
<tr>
<td>III</td>
<td>Virus infection on tumor growth in muscle and in brain</td>
<td>1.0 I.M.</td>
<td>None</td>
</tr>
<tr>
<td>IV</td>
<td>Virus infection on tumor growth in muscle and in brain</td>
<td>1.0 I.M.</td>
<td>None</td>
</tr>
<tr>
<td>V</td>
<td>Virus infection on tumor growth in muscle and in brain</td>
<td>1.0 I.M.</td>
<td>None</td>
</tr>
<tr>
<td>VI</td>
<td>Superimposed injection of uninfected tumor tissue</td>
<td>1.0 I.M.</td>
<td>None</td>
</tr>
</tbody>
</table>

*Where birds received virus only, the day of killing was counted from the day when the other birds in the experiment received the tumor inoculum.†Tumor inoculated into left and virus into right pectoral muscle. ‡Inoculated in opposite breast 3 days later with suspension of uninfected tumor tissue. §Not examined.

lum represented 1.0 per cent by weight of the wet infected tumor tissue. As shown in Table 1, the virus was given either intramuscularly or intracerebrally; by the former route the inoculation site was the pectoral muscle opposite the tumor. Virus was inoculated 3 days after transplantation of the tumor, with the exception of Experiment I, when it was given on the 7th day.

**Virutization.**—In addition to the birds killed for pathology studies in Experiments IV and V (Table 1), others were used for estimation of the virus content of various tissues (Charts 1–5). One bird was used for each determination, and the times of killing are indicated in Charts 1–5. The concentration of virus was determined by giving mice intracerebral inoculations of serial tenfold dilutions of infected tissue in saline, four mice per dilution, and determining the LD₅₀ titer by the method of Reed and Muench (16).

**Experiments.**—Table 1 presents an outline of the nature and purpose of each experiment, the number of birds which were killed and those which were kept under observation, the concentration of tumor tissue in the tumor inoculum, the route of inoculation, and the time when birds were killed for pathological examination. Birds were always killed between 10 and 12 A.M. each day to avoid confusion between physiological (diurnal) and virus-induced alterations of the mitotic rate. In most experiments, virus-infected and uninfected birds were divided into two groups as before (9): the first group was killed as shown in the table, and the second was set aside for observation of tumor development, sickness, and mortality over a period of 3 months.

In view of the recent work of Kidd on the regression of transplanted lymphomas following the inoculation of normal guinea pig serum (2), an additional control experiment was run.

**RESULTS**

**Birds under observation.**—The number of birds which developed palpable tumors and the mortality in each group are indicated in Table 1. The viability of the tumor inoculum was shown by the development of palpable tumors in all uninfected birds inoculated intramuscularly and by the death of all untreated birds which received the tumor intracerebrally. Superimposed inoculation of un-
infected tumor tissue prepared in the same manner as the virus inoculum had no inhibitory effect on tumor growth (Experiment VI).

In all experiments in which the tumor was transplanted into the pectoral muscle, there was a highly significant difference in mortality between the groups which received virus and those which were untreated (Experiments I-IV). Inhibition of growth was so effective in some cases (Experiments II and III) that palpable tumors failed to develop in the virus-infected groups.

There were no deaths and no clinical signs of illness in birds which received virus alone intracerebrally or intramuscularly (Experiments IV and V).

**Transplantation of the tumor in the pectoral muscle.**

a) Pathology: The gross and microscopic changes following transplantation of the RPL-12 lymphoma into the pectoral muscle have already
been described (9). 1 When small amounts of the tumor were transplanted, a number of birds survived the stage of progressive growth, and phagocytosis of tumor cells occurred (9). In the virus-infected birds, the same two phases were recognizable in the life cycle of the implanted tumor cells. The stage of progressive growth, however, terminated 2–3 days after virus inoculation, instead of lasting up to 17 days after transplantation. The onset of the stage of phagocytosis which followed was always related to the time when the virus was given. In all four experiments in which the effects of virus infection on the tumor in the muscle were studied (Experiments I, II, IV, and V), phagocytosis of tumor cells occurred on the 2d and 3d, or the 3d and 4th days after virus injection (Figs. 1 and 2).

Unlike the cells of the uninfected tumor, the tumor cells in the virus-infected birds showed a striking alteration before undergoing phagocytosis: They developed large eosinophilic P.A.S.-positive cytoplasmic inclusions which swelled the cell body, displaced the nucleus to one side of the cell, and even indented the nuclear membrane (Figs. 3A and 3B). Apart from a rare cell showing margination of the nuclear chromatin (Fig. 3B), no further evidence of cellular damage could be detected in the affected cells until they had undergone phagocytosis. The development of the inclusions from the lipochondria of the cell and an analysis of their cytochemical properties are discussed in a succeeding paper (10). After phagocytosis, the tumor cells underwent intracellular degeneration in the same manner as those in uninfected birds (Figs. 1 and 2).

In the virus-infected birds, the host reaction followed a similar but more accelerated course than that already described in uninfected birds (9). When regression of the uninfected tumor occurred, the process was accompanied by extensive local macrophage, lymphocyte and plasma cell infiltration, and by plasmacytic, reticulum-cell, and, later, lymphoid hyperplasia in the spleen (9). The same changes accompanied regression of the tumor after virus infection, except that they began earlier—invariably 2 or 3 days after inoculation of virus.

The only other important differences between the infected and uninfected birds were observed in the central nervous system. The pathological changes were insignificant in the brain and cord of birds bearing the tumor in the pectoral muscle and infected intramuscularly with virus (Experiments IV and V). No important abnormalities were found in any of the four birds killed on the 1st and 2d days after virus inoculation; mild inflammatory changes were present in three of four birds on the 3d day, and in all but one of fourteen birds killed after that. Lesions were present, therefore, from the 3d to the 21st day after inoculation of the virus and were observed in all parts of the gray and white matter and in the leptomeninges of the brain and cord; however, they appeared to be more numerous in the optic tectum and thalamus. The inflammatory changes were essentially vascular and perivascular in distribution and involved venules and capillaries to a greater extent than arterioles. The commonest type of lesion affected the congested venules and arterioles and consisted of infiltration of the vessel wall, Virchow-Robin space, and, to a lesser degree, of the perivascular brain tissue or subarachnoid space by lymphocytes, pleomorphic mononuclear cells, scanty plasma cells, and an occasional lymphoblast or plasmablast. In addition to these vascular changes, there were a few ill-defined foci of lymphocytes, pleomorphic mononucleares, and plasma cells, not obviously related to vessels, but which on serial section were invariably found in the vicinity of one or more dilated capillaries. After the onset of encephalitic changes, the number and character of the inflammatory cells and the nature and severity of the lesions varied little throughout the period of observation. There was no evidence of neuronal degeneration or demyelination.

b) Virology: Virus could be demonstrated in considerable quantity in the pectoral muscle which contained the tumor (Charts 1 and 3). The virus titer reached a peak on the 9d or 8d day after inoculation, at the time when inclusions were present in the tumor cells and phagocytosis was occurring; the titer then decreased until, 8 days later, no virus could be detected (Chart 3). The virus titer in the blood followed the same pattern as that in the muscle, but at a considerably lower level (Charts 1 and 3). Small amounts of virus were demonstrable in the brain on three occasions (Charts 1 and 3)—once on the second and twice on the third day after inoculation, when the earliest histological lesions could be detected in the brain.

Transplantation of the tumor in the brain.—

a) Pathology: The growth of tumor cells in the brain followed a pattern similar to that in the muscle, except that no phagocytosis and virtually no degeneration occurred.

Three days after intracerebral transplantation, moderate numbers of tumor cells and a few macro-
phages and lymphocytes could be seen in the subarachnoid space and occasionally discretely scattered in the brain substance near the site of the needle track. During the next few days, large numbers of tumor cells packed the subarachnoid space and ventricles and infiltrated the brain substance discretely from the site of inoculation; they extended along the Virchow-Robin spaces of the blood vessels and finally produced a diffuse widespread infiltration of the brain, the choroid plexus, and even the spinal cord as far caudal as the lumbo-sacral region (Figs. 4 and 5).

After the initial short period of phagocytosis of the nonviable debris of the inoculum, the local host reaction was limited throughout to minimal lymphocytic cuffing of a rare vein. The striking changes in the spleen which accompanied the same period of growth of the tumor in the pectoral muscle were not observed when the tumor was transplanted into the brain.

On the 2d or 3d day after inoculation of the virus by the intracerebral or intramuscular route (Experiments III, IV, and V), cytoplasmic inclusions could be detected in many of the tumor cells (Fig. 6). During the next few days the inclusions increased in size and were found in nearly every tumor cell, including many which were in mitosis. Mitotic figures, and in particular metaphase plates, were very conspicuous. Whereas a binucleate cell was only rarely observed in the uninfected tumor, the number of these cells was definitely increased after the appearance of cytoplasmic inclusions. A more extensive analysis of mitosis and other cytological features will be presented in our next communication (10). As in the muscle, the appearance of inclusions coincided with the onset of phagocytosis. In the subarachnoid space and ventricles, the ingestion of tumor cells was neither so extensive nor so rapid as in the muscle, and a few still remained free until the death of the bird. In the brain and Virchow-Robin spaces, phagocytosis was even more deficient, and many tumor cells with large cytoplasmic inclusions were not ingested.

In all three experiments (Experiments III, IV, and V) the local host reaction in the brains of the virus-infected birds was indistinguishable from that of the uninfected tumor-bearing controls until the 2d day after inoculation of virus. At this point, macrophages and scanty lymphocytes began to appear around the venules and arterioles in the subarachnoid space, and, to a lesser degree, in the brain. The inflammatory cells infiltrated more widely, and the macrophages in particular increased considerably in number during the succeeding days; but the changes were almost entirely limited to the areas of tumor cell infiltration. By the 4th or 5th day after virus inoculation, the host reaction in the brain was never more developed than that which occurred on the 2d or 3d day when the tumor was grown in the muscle. Similarly, the splenic host reaction was delayed in all three experiments (Experiments III, IV, and V) and did not develop until the 4th or 5th day after virus infection. All the birds died in the next day or two, and changes were limited to the plasmacytic hyperplasia which characterized the early stage of the splenic reaction observed when the tumor was grown in the pectoral muscle.

In two out of ten birds killed on the 4th and 5th days after virus infection, an additional lesion was present in the brain. In areas where tumor cells were abundant and contained prominent inclusions, a few venules showed a peculiar fibrinoid degeneration (Fig. 7). The fibrinoid change was associated with disruption of the elastic and reticulin fibers and was accompanied by considerable recent hemorrhage and neutrophil polymorphonuclear inflammatory cellular infiltration of the vessel walls and surrounding tissues.

b) Virology: Irrespective of the route of inoculation, the virus was demonstrable in considerable amounts in the brain containing the tumor (Charts 2 and 3). The maximum amount of virus was present on the 2d-4th day after virus inoculation, and, in Experiment V, in which the birds lived to the 5th day (Chart 3), a slight drop occurred. Again, the high levels of virus corresponded with the period in which large numbers of the tumor cells contained inclusions. The blood virus level reached a lower titer on the 2d day after inoculation by either route, but fell off more rapidly when the virus was administered intracerebrally (Charts 2 and 3).

Effect of tumor on virus multiplication and pathogenicity for host.—In the absence of a tumor, the virus could not be demonstrated in the blood after intracerebral, nor in the brain after intramuscular, inoculation of the virus (Charts 2 and 3). The virus was detectable in minimal amounts on one occasion only in each experiment—in the brain of a bird inoculated intracerebrally 5 days previously, and in the blood of a bird inoculated intramuscularly 2 days before.

When the tumor was transplanted into the brain 3 days before intracerebral inoculation of virus, the LD₅₀ titer in the brain continued to rise to a peak of more than 10⁻⁴ on the day before death (Chart 2). In the blood, after reaching a lower peak on the second day, the amount of virus dropped slowly but remained detectable through-
out. Similarly, the amount of virus demonstrable in the brain after intramuscular inoculation of virus was greatly enhanced by the presence of the tumor in the brain or in the muscle (Chart 3); the virus titer in brain was never higher than that in blood except when the tumor was growing in the brain (Chart 3). The greatest concentration of virus was always found in the tissue containing the tumor (Charts 1–3). The presence of the tumor greatly increased the amount of virus in the tissue in which it grew and also secondarily elevated the virus content of the blood and other tissues. In this sense, therefore, virus multiplication in the presence of the tumor.

The effect of growth of tumor in the brain on the pathogenicity of virus given by the intracerebral route was shown by comparing the inflammatory reaction following administration of virus alone, tumor alone, and of tumor followed by virus (Experiment IV). Similarly, the effects of the presence of tumor in the brain and in the muscle on the pathogenicity of virus inoculated intramuscularly were demonstrated in Experiment V. Except when the tumor was grown in the brain, the nature of the encephalitis in all groups was similar to that already described. The presence of a tumor in the brain of a bird infected with virus intracerebrally or intramuscularly resulted in a more severe inflammatory reaction than was produced by the virus alone; the lesions, however, were limited to the areas of tumor cell infiltration. The severity of the inflammation cannot be accounted for by a summation of the individual effects of virus or tumor. When the tumor was grown in the muscle, subsequent intramuscular inoculation of virus produced a very slight increase in the severity of the resultant encephalitis over that produced by virus alone. Encephalitic lesions were invariably present on the 1st and 2d days after inoculation of virus alone by any route. The presence of a tumor in brain or muscle delayed the onset of encephalitic changes by 1 and 2 days, respectively.

**DISCUSSION**

N.F.T. virus multiplies in the RPL-12 lymphoma and is capable of producing regression and a highly significant reduction in the mortality of chickens bearing the tumor in the pectoral muscle. Virus infection results in the production of specific changes in the tumor cells which do not occur in uninfected controls. These changes do not appear to lead to actual disintegration of the tumor cells but render them susceptible to phagocytosis by the macrophages of the host. Phagocytosis of uninfected tumor cells in the muscle has been shown to occur at a much later stage if the birds survive (9). The onset of phagocytosis in the uninfected bird is preceded by plasmacytic and reticulum-cell hyperplasia in the spleen, which has been interpreted as the morphological manifestation of an immune reaction (9). A similar phenomenon is coincidental with, but does not precede, the onset of phagocytosis of tumor cells in the muscle in the virus-infected birds. Since virus infection alone does not produce these changes, the virus-infected tumor cell would appear to be more antigenic than its uninfected counterpart. Hence, the host defenses may play an important part in causing the regression of the tumor.

Confirmation of this hypothesis is provided by the results of transplantation of the tumor in the brain. Here virus infection is incapable of saving the bird, and there is considerable evidence that the host defenses against the growth of the tumor are deficient. Phagocytosis of tumor cells in the brain and splenic host reaction do not occur in the uninfected controls, and both processes are considerably reduced and delayed even after virus infection. If, as we have attempted to show in our previous work (9), the splenic reaction is an indication of an immune reaction, uninfected RPL-12 tumor cells in the brain may be less effective in inducing an antibody response than those in the muscle. On the day before death, however, although many tumor cells still persist in the brains of virus-infected birds, phagocytosis is fairly extensive, and the earlier stages of plasmacytic hyperplasia are quite definite in the spleen. Virus infection may increase the antigenic potency of the tumor cell or break down the barrier which otherwise prevents the initiation of the splenic response. Since the virus-infected birds die before the uninfected controls, it seems probable that they succumb to the severe inflammatory changes involved in the removal of tumor cells rather than to the effect of tumor growth alone.

Whatever the route of tumor transplantation and of virus inoculation, the highest concentration of virus is always present in the tissue containing the tumor. The virus has, therefore, a stronger affinity or tropism for tumor than for any other tissue in the chicken. The absence of encephalitic changes during the 1st day or two after inoculation of virus, when the tumor is present in brain or muscle, suggests that the attraction of the virus for the tumor is so great that relatively nonpathogenic amounts reach the brain tissue at this time. If this is true, it may be possible to infect a tumor with a highly “oncotropic” virus and destroy any free virus with antiserum or an antiviral agent.
before it has time to initiate a pathogenic infection of the normal host tissues.

After the 2d or 3d day, when the pathogenic effects of the virus on the tumor become manifest, virus is released into the blood stream, and the resultant pathological changes are somewhat more severe than those produced by inoculating the virus into an intact bird. When the tumor is grown in the muscle of the chicken, the encephalitic process produced by inoculation of virus is still insignificant and does not produce any clinical manifestations of illness. The problem is more complicated in the brain, when it is both the site of the infected tumor and the only tissue susceptible to damage by the virus. There is some evidence, however, that, apart from the increased inflammatory reaction in the immediate vicinity of the tumor, the changes in other parts of the brain are less severe than those following intracerebral inoculation of virus alone. In this case a differential tropism may exist between brain and tumor, whereby the greater avidity of the tumor for the virus may protect the normal brain tissue.

SUMMARY

1. When the RPL-12 chicken lymphoma is transplanted into the pectoral muscle of chickens, superimposed infection with the N.F.T. strain of St. Louis encephalitis virus induces regression and a significant reduction in mortality. Infection with the virus does not produce oncolysis when the tumor is transplanted into the brain.

2. N.F.T. virus multiplies in the tumor but does not destroy the tumor cells; it produces specific changes in the cells which (a) render them susceptible to phagocytosis and (b) increase their ability to stimulate the local and splenic defense mechanisms of the host. The failure of virus infection to induce regression when the tumor is grown in the brain is attributed to a deficiency of the host defenses.

3. The inflammatory changes in the brain after the inoculation of N.F.T. virus are accentuated, but their onset is delayed 1 or 2 days by the presence of the RPL-12 tumor in the brain or muscle, respectively. When the tumor is grown in the muscle, the lesions produced by virus infection are still, however, inconspicuous and unaccompanied by any clinical signs of illness.

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REFERENCES

Fig. 4.—Extensive infiltration of subarachnoid and Virchow-Robin spaces and brain substance by tumor cells 6 days after intracerebral inoculation of tumor. H & E, × 200.

Fig. 5.—Massive infiltration of lateral ventricle (top right) and adjacent brain substance by tumor cells. H & E, × 950.

Fig. 6.—Large cytoplasmic inclusion in tumor cell in the brain 4 days after inoculation of virus. H & E, × 1800.

Fig. 7.—Fibrinoid degeneration of venule in brain. H & E, × 950.
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