Recent observations in this laboratory on the mechanism of virus oncolysis have been largely confined to a study of the effects of virus infections on transplantable tumors (6, 11). The relative importance of the so-called oncolytic agent and the contribution of the host defences in bringing about regression of a transplantable tumor are difficult to assess. In the course of our work with the RPL-12 transplantable lymphoma of chickens, we have confirmed the observations of Olson (10) and Burmester and Prickett (2) that, in serial passage, the inoculation of varying amounts of this tumor could lead to absorption of the inoculum without tumor formation, growth of a palpable tumor with subsequent regression, or growth progressing to the death of the bird. A study of the histological changes during regression of this tumor following infection with an oncolytic virus\(^1\) revealed that phagocytosis of tumor cells plays an important part. It seems desirable, therefore, to assess the nature of the host response to transplantable tumors before attributing regressive changes to virus infection. For this reason, the following experiments were performed.

MATERIALS AND METHODS

Birds.—Except where otherwise stated, 2- to 3-week-old New Hampshire Red chicks were used. All birds were vaccinated against Newcastle disease when 1 day old.

Tumor.—The RPL-12 tumor used in these studies was originally transplanted by Olson (10) from a female cross-bred (Rhode Island Red female × Plymouth Rock male) chicken; it was obtained through the courtesy of Dr. B. R. Burmester of the Regional Poultry Research Laboratory, East Lansing, Michigan, and serially passed in the pectoral muscle. For the preparation of the inoculum, tumors were excised 7 days after transplantation. Pieces of tumor, as free as possible from muscle and necrotic material, were ground up in a Ten Broeck grinder (12) and suspended in physiological saline. Birds were given inoculations in the pectoral muscle of 0.25 ml. of these suspensions, made to various concentrations as indicated in the respective protocols.

Biological procedures.—In most experiments, many of the birds were sacrificed for examination, and the remainder were set aside as controls to assess the ability of the inoculum to produce lethal or spontaneously regressing tumors; the mortality was noted daily for a period of 3 months, and the development of tumors was determined by palpation of the pectoral muscle 3 times a week.

The concentration of tumor tissue in the inoculum, the number of birds in the control and experimental groups, the time of sacrifice, and the purpose of each experiment are shown in Table 1. Twenty control birds which survived implants of heat-killed tumor in Experiment II were subsequently tested for susceptibility to living tumor cells by the injection of a 10 per cent suspension of fresh tumor tissue into the opposite breast 3 weeks later.

To obtain a high rate of regression a small concentration of tumor was used in the inoculum for Experiment IV (Table 1). In Experiment IV-A birds were sacrificed when the tumor showed clinical evidence of regression, with the exception of the first four, which were sacrificed to observe the earlier growth of the tumor. In the second part of the experiment (IV-B), most of the birds were sacrificed when the tumors were large, and before they showed clinical evidence of regression. In Experiment IV-C, birds were sacrificed mainly between the 7th and 17th day after inoculation—the period when the most interesting histological changes were expected.

In Experiment V-A, a group of 73-day-old birds which had survived the inoculation of a 0.01 per cent tumor suspension at the age of 10 days were inoculated in the opposite breast with a 10 per cent suspension of tumor tissue. Experimental and control groups were sacrificed and observed as before (Table 1). In addition, the potency of the second inoculum was tested by the simultaneous inoculation of seventeen birds...
### TABLE 1
Experimental Data and Summary of Histological Changes at Site of Inoculation

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Inoculum</th>
<th>Experimental (Birds Sacrificed)</th>
<th>Controls (Birds Set Aside for Observations)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per Cent Tumor Suspension</td>
<td>Days after tumor implantation</td>
<td>Total (n.s.)</td>
</tr>
<tr>
<td>I Early stages of tumor growth</td>
<td>2.0</td>
<td>5 5 2</td>
<td>100</td>
</tr>
<tr>
<td>II Host reaction to heat killed tumor</td>
<td>14 NG</td>
<td>1 2 2 2 2 1</td>
<td>5 5 2</td>
</tr>
<tr>
<td>A Course for lethal tumor</td>
<td>10.0</td>
<td>1 1 1 1 2</td>
<td>8 8 7</td>
</tr>
<tr>
<td>B Spontaneous regression after first inoculation of tumor</td>
<td>0.1</td>
<td>1 1 1 1 2</td>
<td>1 1 1</td>
</tr>
<tr>
<td>C Response to second inoculation of tumor</td>
<td>0.1</td>
<td>2 1 2 2 2 1</td>
<td>1 1 2</td>
</tr>
</tbody>
</table>

NG = No growth of tumor cells.

* Numbers indicate number of birds sacrificed at that stage.

PG = Stage of progressive growth.

P = Stage of phagocytosis.

RT = Stage of restitution of muscle.

† Sacrificed 1, 2, 3, 6, 7, 9, 11, 13, and 19 hours after inoculation.

‡ Found dead.
of the same age, which had not previously received injections of tumor; all developed tumors and died. In preparation for the latter part of this experiment (V-B) a 5 per cent suspension of tumor was inoculated into a group of 16-day-old birds. Regression of the tumor was brought about by treatment with an oncolytic virus 3 days later (11). When 75 days old, these birds were challenged with a 10 per cent suspension of tumor tissue and divided into control and experimental groups as before (Table 1). The potency of the second inoculum was confirmed by the simultaneous inoculation into nine birds of the same age which had not previously received injections of the tumor: all developed tumors, and six died.

The normal histology of birds of comparable age to the experimental groups was determined in twelve chickens sacrificed at the rate of three a week from the age of 3 weeks.

Pathology procedures.—All birds of the experimental groups were killed with chloroform and autopsied. Blocks were taken from both pectoral muscles, skeletal muscle of thigh, sciatic nerve, spleen, femoral bone marrow, thymus, bursa of Fabricius, liver, pancreas, esophagus, proventriculus duodenum, ileum, large intestine, cecum, thyroid, suprarenal, gonad, kidney, heart, larynx, trachea, and lung. For routine purposes, tissues were fixed in formal sublimate, and paraffin sections were stained with hematoxylin and eosin. Where finer detail was required, sections were stained by Barrett's bone marrow method (1). Six to ten blocks of the injected muscle were prepared in the first instance, and, where the tumor could not be detected, serial sections were made.

RESULTS

Controls.—The viability of the inoculum was confirmed by the development of palpable tumors in all controls receiving the first inoculation of tumor. In the experiments designed to produce a fatal outcome (Experiments III-A and B), 87 per cent and 67 per cent of the birds died. In the group in which spontaneous recovery was attempted, 46 per cent of the controls survived (Experiment IV-B, Table 1). Resistance to a second inoculation of tumor was shown by the fact that all birds survived reinoculation with a heavy dose of viable tumor (controls, Experiment V), whereas 23 of 26 birds of the same age died when they were given the same inoculum for the first time. All birds which had previously been given heat-killed tumor developed tumors, and all died after challenge with viable tumor.

When the tumor was lethal, most of the birds died within 14 days and none later than 26 days after inoculation. The birds that died had massive tumors extending the whole length of the sternum with extensive edema of the surrounding tissues and occasional secondary deposits in the proventriculus, pancreas, lung, heart, and gonads.

Many birds of the normal control and of the experimental groups showed signs of mild chronic respiratory disease, but this was equally common in both groups.

Experimental: Histological changes in the muscle following the first inoculation of tumor.—During the first 48 hours after inoculation of the tumor, the perimysial, and to a lesser extent the endomysial spaces of the pectoral muscle, contain linear aggregations of eosinophilic granular material and degenerating muscle fibers mingled with nuclear fragments and scanty primitive cells resembling lymphoblasts (Fig. 1). Since these later come to comprise the vast majority of cells in the tumor (Figs. 2 and 3), they will be designated tumor cells. They are large, round cells 9–15 μ in diameter; each has a prominent nucleus which is more or less centrally placed in the moderately basophilic cytoplasm. The nucleus is round, oval, or slightly indented, and has a well defined nuclear membrane and a large nucleolus (Fig. 8). Occasional tumor cells in mitosis are present as early as 3 hours after implantation. 2

Around the linear collections of debris and tumor cells there are moderate amounts of hemorrhage, edema, and inflammatory cellular reaction consisting of neutrophil polymorphs, macrophages, and a few small lymphocytes. At first, the inflammatory cells are confined to the vicinity of small veins, but within a few hours after inoculation they mingle with the debris of the inoculum. Throughout this period, increasing numbers of lymphocytes appear around the small veins in the surrounding muscle (Fig. 1).

The possibility of the inflammatory reaction being the result of differentiation of the lymphoblastic tumor cells or of virus activity in the inoculum was excluded by examining sections of muscle after the inoculation of heat-killed tumor tissue. The reaction in this case is identical to that observed with the living tumor cells, except that macrophages are more numerous, and occasional foreign body giant cells are found; no large lymphoblastic cells are present in the necrotic debris (Fig. 4).

Forty-eight hours after inoculation, tumor cells can be observed in the perimysial and endomysial spaces at some distance from the debris of the inoculum which appears to be undergoing phagocytosis by the macrophages. Four days after implantation the nonviable remnants of the inoculum are no longer recognizable, and there is considerable infiltration of the perimysial and endomysial spaces by tumor cells showing numerous mitotic figures. Tumor cells are commonly found in the wall and lumen of veins and in the lymphatics of the muscle.

The tumor continues to grow for the next 6–13 days; it now comprises a large central zone of muscle fibers widely separated by sheets of closely packed tumor cells (Fig. 2) and a periphery where

*A study of the cytochemistry of these cells will be presented in a subsequent paper.
cells which survive around the anterioles in the areas of central necrosis. By the time these cells are affected, there is extensive diffuse inflammatory cellular infiltration of the widely separated muscle fibers which were previously the site of the tumor. In addition to macrophages and increasing numbers of lymphocytes, large numbers of plasma cells appear in the exudate.

After the 20th day in all birds, and occasionally before this, tumor cells are no longer present, and the process of restitution of the muscle begins (stage of restitution of muscle, Table 1). Numerous lymphocytes and lymphoblasts accumulate around the veins, and germinal centers develop. Syncytial masses of sarcoblasts, new capillaries, macrophages, and scanty fibroblasts invade the necrotic areas. Meanwhile, the diffuse inflammatory cellular reaction in the rest of the muscle diminishes. Finally, the inflammatory exudate is absorbed, the muscle fibres are largely restored, and the lymphoid hyperplasia subsides. A month to 6 weeks after inoculation, the only abnormality in the pectoral muscle consists of a few foci of lymphocytes (Fig. 10) and occasional minute areas of fibrosis.

**Histological changes in other organs and tissues.**—
The only important abnormalities in the other organs and tissues are as follows: (a) direct extension of the tumor into the neck and through the skin, (b) numerous tumor cells in the capillaries of the lung, liver, or spleen, (c) secondary growths in the proventriculus, pancreas, lung, heart, and gonads, and (d) changes in the spleen.

Direct extension into the neck is common, and ulceration of the skin is produced by the largest tumors. In three out of 115 birds the capillaries of the lung, liver, and spleen are packed with tumor cells. Secondaries occur only in birds sacrificed, or dying, between the 10th and 20th days after implantation of the tumor, and of 48 birds in this category approximately one-quarter have histological evidence of secondary growth. In all birds in which the primary tumor is regressing, similar changes, including extensive phagocytosis of tumor cells, and lymphoid hyperplasia, are present in the secondary deposits.

The changes in the spleen are of extreme interest. The normal chicken spleen (Fig. 12) is essentially similar to that of mammals. The white pulp is arranged eccentrically around the arterioles, and occasional well defined lymph follicles are present. The sheathed arteries are surrounded by three or four layers of concentrically arranged reticulum cells and a small outer zone of mononuclears. The red pulp contains a high proportion of lymphocytes, occasional lymphoblasts, plasmablasts,
plasma cells, mast cells, and the formed elements of the peripheral blood.

During the stage of progressive growth of the tumor, there is an increase in the number of plasmablasts and plasma cells in the pulp, especially around the sheathed arteries (Fig. 14). Towards the end of this phase this is accompanied by a hypertrophy and hyperplasia of the reticulum cells of the sheathed arteries (Fig. 14). Some of the more peripheral reticulum cells become rounded off and develop hyperchromatic nuclei with a prominent nucleolus and deeply basophilic cytoplasm. All gradations from these cells to mature plasma cells can be observed.

With the onset of phagocytosis in the tumor, there is congestion of the pulp and sinuses and depletion of the lymphoid tissue. The peripheral reticulum cells and mononuclears of the sheathed arteries are separated by blood channels and incorporated into the red pulp. Both pulp and sinuses now contain large numbers of mononuclear cells. Meanwhile, the plasma cells continue to increase in the red pulp.

By the time all the tumor cells have disappeared and large numbers of lymphoid and plasmacytic cells are present in the muscle, the sheathed arteries are reduced to normal or subnormal proportions, lymphocytes reappear in considerable numbers, the white pulp increases in size, and prominent germinal centers develop (Fig. 15). The plasmacytic cells in the spleen steadily diminish in number, and finally the lymphoid hyperplasia subsides. Four to 6 weeks after inoculation, when the pectoral muscle is restored and only a few lymphoid foci remain at the site of the tumor, the spleen is no longer distinguishable from that of a normal bird of the same age.

There are considerable variations in the amount and character of the lymphoid tissue in the rest of the body of the normal and experimental birds, but it is not possible to detect any consistent alterations comparable to those observed in the spleen.

The spleens of birds inoculated with heat-killed tumor do not show these changes and, indeed, show no significant abnormality.

**Histological changes following the second inoculation of tumor into birds surviving the first implantation.**—In the pectoral muscle the stages of progressive growth, phagocytosis of tumor cells, and restitution of muscle are essentially the same as after the first inoculation of tumor. The tumors, however, are much smaller, and the onset of plasmacytosis is earlier (Experiment V, Table 1). Tumor cells are not found in the pectoral muscle later than the 11th day after the second inoculation. Secondary growths do not occur.

### TABLE 2

<table>
<thead>
<tr>
<th>Duration (DATE)</th>
<th>Tumor Cells</th>
<th>Local Host Reaction</th>
<th>Host Reaction</th>
<th>Remote-Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-7</td>
<td>Progressive growth</td>
<td>Phagocytosis of non-neoplastic debris of inoculum. Some lymphocytic reaction.</td>
<td>No changes</td>
<td>No changes</td>
</tr>
<tr>
<td>14</td>
<td>Absent</td>
<td>Diminishing lymphocytes and plasma cells.</td>
<td>Return of lymphoid tissue and plasma cells to normal.</td>
<td>Return of lymphoid tissue and plasma cells to normal.</td>
</tr>
</tbody>
</table>

Plasmacytic and lymphoid changes in the spleen follow the same pattern in relation to the muscle pathology. The plasma cell increase is less striking, begins earlier, reaches a peak in the late stage of phagocytosis, and is no longer detectable 3 weeks after inoculation. Definite reticulum-cell hyperplasia was present in two birds only, on the 8th and 10th days after challenge. Lymphoid hyperplasia of moderate degree is limited to the early stages of restitution of the muscle.

**DISCUSSION**

The first inoculation of tumor results in two main processes: growth of tumor cells and host reaction, which are summarized in Table 2. Despite the presence of occasional macrophages the tumor cells are not susceptible to phagocytosis and produce minimal local reaction during the first 10 days after inoculation. Between the 10th and 21st days a change occurs in the tumor cells whereby they become susceptible to phagocytosis and provoke an extensive local inflammatory response. The phenomenon is essentially similar to the opsonin effect of antibodies on bacteria.

The host has overcome invasion by tumor cells.
Furthermore, regression of the neoplasm is followed by a state of increased resistance to a second inoculation of tumor in a different site. Such a phenomenon is strongly suggestive of the presence of circulating antibody.

The association of plasma cells, lymphocytes, and reticulum cells with the production of antibodies is well known. In a recent review of this subject Delaunay (3) implicates all three types of cells, although other workers (4, 5, 9) favor the plasma cell in particular. Makinodan et al. (8) recently described plasmacytic hyperplasia in the spleens of chickens developing precipitins to bovine serum albumen. Ehrich and his co-workers (4) observed a plasmacytic, followed by a lymphocytic, hyperplasia in the lymph nodes of rabbits during the development of antibodies to typhoid vaccine. Since the lymphocytic reaction was maximal after the antibody formation had passed its peak, they concluded that the plasma cell and not the lymphocyte was responsible for antibody production.

Throughout the stage of progressive growth of this tumor there is a hyperplasia of the plasma cell series and later of their precursors, the reticulum cells of the sheathed arteries in the spleen. Just before the plasma cells attain their maximum number in the spleen, phagocytosis of tumor cells occurs. Such changes do not occur in birds inoculated with heat-killed tumor which are not resistant to subsequent challenge with tumor. It would seem reasonable, therefore, to suggest that the plasma cells contribute to the production of antibodies.

The role of the lymphoid tissue is more difficult to assess. There are no changes in the splenic lymphoid tissue during the stage of progressive growth of the tumor, and the small amount of lymphocytic reaction in the muscle remains constant throughout this phase and does not seem to interfere with the proliferation of the tumor cells. A similar local reaction occurs after the inoculation of heat-killed tumor, which does not produce immunity. Phagocytosis of tumor cells begins before the onset of extensive local lymphocytic infiltration. The transient depletion of lymphocytes in the spleen at this time may be the result of lysis of lymphocytes with liberation of antibody, or may represent the mobilization of lymphocytes to the muscle. If the former explanation is true, the final stage of local and splenic lymphoid hyperplasia could be a compensatory replacement phenomenon.

Reticulum-cell hyperplasia in the spleen occurs in the later stages of progressive growth of the tumor and undoubtedly contributes to the formation of plasmablasts. The majority of the reticulum cells, however, become rounded off towards the periphery of the sheathed arteries and form monocytes or macrophages. Hence, the onset of macrophage infiltration of the tumor coincides with a sudden depletion of the reticulum cells and mononuclears in the sheathed arteries of the spleen.

**SUMMARY**

1. Inoculation of varying amounts of the RPL-12 lymphoma can lead to growth of a palpable tumor with subsequent regression or to growth progressing to the death of the bird.
2. Regression of the tumor is followed by resistance to subsequent challenge with the same tumor, which is greater than that of normal birds of the same age.
3. Two phases are recognizable in the life cycle.
Fig. 6.—Macrophage infiltration around a vein in the center of the tumor. Note intact tumor cells at top and bottom. Barrett’s stain. ×760.

Fig. 7.—Several macrophages containing tumor cells near edge of tumor. Barrett’s stain. ×950.

Fig. 8.—Degenerating tumor cells within macrophages, and apparently undamaged tumor cells nearby. Barrett’s stain. ×1,620.

Fig. 9.—Nucleolar swelling and margination of chromatin of tumor cell inside a macrophage. H and E stain. ×1620.

Fig. 10.—Main nucleus and a small micronucleus formed by a tumor cell undergoing intracellular degeneration. H and E stain. ×1620.

Fig. 11.—Perivenous (top) and diffuse infiltration of regenerating muscle by lymphocytes and plasma cells, after phagocytosis of tumor cells. H and E stain. ×300.
Fig. 12.—Normal chicken spleen showing pale staining reticulum cells and mononuclears of sheathed arteries, and numerous lymphocytes in the red pulp. H and E stain. ×300.

Fig. 13.—Large numbers of plasmablasts and plasma cells in splenic pulp during late stage of progressive growth of tumor. H and E stain. ×1620.

Fig. 14.—Spleen during stage of phagocytosis of tumor cells in the muscle. Note greatly enlarged sheathed arteries and depletion of lymphocytes in pulp. H and E stain. ×300.
FIG. 15.—Conspicuous germinal centers and small sheathed arteries in spleen during early stage of restitution in muscle. H and E stain. ×300.

FIG. 16.—Small lymphoid focus in pectoral muscle 6 weeks after inoculation of tumor. H and E stain. ×150.
of the implanted tumor cells. The first, a stage of progressive growth, is of 10–17 days’ duration. The second, a stage of regression, is characterized by phagocytosis of apparently intact tumor cells, and is of 3–4 days’ duration.

4. The nature of the local and distant host reaction to the implanted tumor is described, and the changes which occur in the lymphoid and plasma cell series are interpreted as the morphological manifestations of an immune reaction.

5. Inoculation of heat-killed tumor tissue is not followed by the changes in lymphoid and plasma cells which accompany a progressively growing tumor and does not give rise to any resistance to subsequent challenge with live tumor cells.

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Robert Love and George R. Sharpless


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