Enhanced Syngeneic Tumor Destruction by *In Vivo* Inhibition of Suppressor T Cells Using Anti-I-J Alloantiserum

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The cellular response to a methylcholanthrene-induced sarcoma S1509a has been investigated. Histologic analysis of the *in vivo* response to S1509a included a study of tumor development in nonimmune, tumor immune, or hyperimmune syngeneic mice, as well as in nonimmune animals treated with antiserum produced to interact solely with determinants encoded by the I-J subregion of the H-2 major histocompatibility complex. Tumors from immune or hyperimmune mice showed marked infiltration by mononuclear and, to a lesser extent, polymorphonuclear cells, with marked tumor cell necrosis. Anti-I-J treated mice displayed similar but quantitatively reduced leukocytic infiltrates and less evidence of tumor cell degeneration. Untreated nonimmune mice, on the other hand, revealed only mild leukocytic infiltration with little or no necrosis of the tumor. Thus, the administration of anti-I-J antiserum, which has been shown to diminish suppressor cell activity, is associated with increased leukocytic infiltration and enhanced syngeneic tumor destruction *in vivo* in the nonimmune host. (Am J Pathol 92:491–506, 1978)

SUPPRESSOR THYMUS-DERIVED (ST) CELLS which specifically limit the primary effector response to syngeneic methylcholanthrene-induced tumors [1] arise in the lymphoid organs of murine tumor-bearing hosts (TBH). ST cells inhibit the rejection of a secondary tumor challenge when adoptively transferred to syngeneic immune recipients.[1–4] Characterization of ST cells has shown them to bear I-J determinants encoded by the H-2 major histocompatibility complex (MHC); to be θ-bearing, ATS-sensitive, cortisone-resistant, and light in terms of density; and to reside within the lymph nodes, spleen, and thymus of TBH.[3] ST cells are detectable within 24 to 48 hours after exposure to tumor antigen. However, these cells disappear from the primary TBH within 5 days of tumor removal.[3]

ST cells have been shown to elaborate tumor antigen-specific suppressor factors (SF) which have the capacity to limit host responses in a manner analogous to that of suppressor cells.[4,5] Tumor-specific SF have

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biologic and immunochemical properties similar to those of other specific suppressor factors capable of inhibiting antibody responses,[6-8] allotype specific responses,[9] or contact sensitivity reactivities.[10,11] Tumor-specific SF further resemble other described suppressor factors in that they also appear to bear determinants encoded by the I-J subregion of the MHC.[12]

We have previously studied the effects of anti-I-J alloantiserum on the response of nonimmune A/J mice to a variety of syngeneic methylcholanthrene tumors.[1] Treatment with anti-I-J antiserum inhibits the growth of primary tumors, presumably by limiting the development of suppressor T-cell activity within these animals, since suppression could no longer be adoptively transferred with cells of anti-I-J treated animals to immune recipients.

We now extend these studies to document histologically that treatment with anti-I-J antiserum causes enhanced tumor destruction throughout the period during which it is administered, presumably as a consequence of augmenting host effector responses by eliminating suppressor T-cell influence. We have examined the effect of treatment with anti-I-J antiserum on various organs in the primary tumor-bearing host.

Materials and Methods

Mice

A/J (H-2^a) female mice 8 to 10 weeks of age were obtained from Jackson Laboratories, Bar Harbor, Maine.

Tumors

The S1509a tumor, a methycholanthrene-induced sarcoma in A/J mice, was maintained both *in vitro* and *in vivo* by transfer of ascitic fluid obtained from the peritoneal cavity of mice given 10^6 sarcoma cells.[1-3] Culture conditions have been described previously.[1-3]

Antiserum

The antiserum used in these experiments included Batch 480 of (3R × DBA/2)F_1 anti-5R or Batch 482 of 3R anti-5R, both of which are hereafter designed anti-I-J^k. These antiserum are capable of removing I-J^k–coded suppressor factors.[8] As controls, normal A/J serum was used. In all experiments, mice received daily intravenous injections of 0.2 ml containing 2 μl of neat serum, an amount that has been shown to limit tumor growth *in vivo*.[1]

Procedure for Immunization

For immunization with the S1509a tumor, A/J mice received 10^6 cells subcutaneously in the back. The tumor was removed surgically 1 week later. Such mice are regarded as immune, as evidenced by their rejection of a subsequent tumor challenge. Hyperimmune mice were prepared by inoculating immune mice with several subsequent injections of live tumor cells.[1-4] Animals treated in this manner consistently can reject a 10^6 cell inoculum of S1509a within 14 days.

Tumor Assay

After inoculation of 10^6 tumor cells subcutaneously, mice were randomly assigned to experimental and control groups. Beginning on the third day after inoculation, hair was removed over the tumor site with a chemical depilating agent (Neet, Whitehall Lab, N.Y.). The tumors were examined independently by two experimenters, and tumor diameters were measured with vernier calipers at right angles to calculate tumor size in square centimeters.

Histologic Analysis

The tumor and a wide margin of adjacent skin were excised. Two sections of each tumor were examined. In Experiment 1, 1 or 2 randomly chosen animals in each group (non-immunized controls given normal A/J serum; anti-I-J^k treated; immunized; hyper-immunized) were killed at Days 3, 7, and 10. The thymus, liver, kidneys, spleen, and draining lymph nodes from each animal were also examined. In Experiment 2, tumors were removed from 4 anti-I-J^k treated animals and four control mice on Day 8 after tumor inoculation; other organs were not examined. All tissues were fixed in buffered 10% formalin (Fisher Scientific, Fairlawn, N.J.). Histologic sections were prepared by Ms. M. Hagney of Harvard Medical School. Sections were cut at 4 μ and stained with hematoxylin and eosin.

Statistical Analysis

The statistical significance of the results obtained by measurement of tumor size was calculated by the Student t test as computed by the Wang programmable computer. The arithmetic means and standard errors of the means are indicated in the figures.

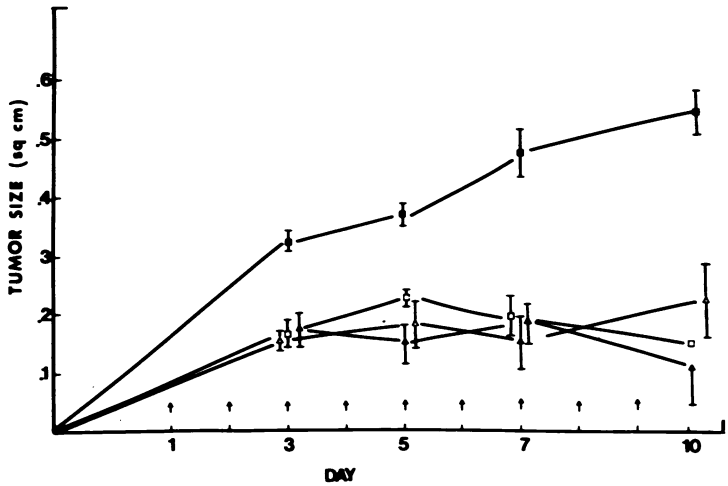
Results

Effect of Anti-I-J Alloantiserum on the Primary Immune Response to S1509a

We previously demonstrated that anti-I-J^k alloantiserum administered intravenously at doses of 2 to 20 μl at the time of 10^5 to 10^6 S1509a tumor cell inoculum and daily thereafter could significantly limit tumor growth *in vivo*.[1] The ability of 2 μl of anti-I-J^k alloantiserum administered in this manner to limit growth of a 10^6 S1509a tumor challenge is depicted in Text-figure 1. The effect becomes manifest as early as the fourth day and persists for the duration of antiserum therapy. Also shown are the growth characteristics of a 10^6 S1509a cell inoculum in S1509a immune and hyperimmune A/J mice. As is clearly seen, the limited tumor growth pattern resulting from anti-I-J^k antiserum therapy is similar to that seen in the immune or hyperimmune host.

Histologic Analysis of Tumors and Lymphoid Organs of Animals Treated With Anti-I-J Antiserum

A few general comments will be made about the tumor and the reaction to it before the individual groups are analyzed. The tumor was composed of large polygonal cells (Figure 1) which usually grew in syncytial form as a single nodule, most often in the subcutaneous tissue. In some sections,



TEXT-FIGURE 1—10^6 S1509a cells were inoculated subcutaneously into syngeneic nonimmune A/J which received 2 μl/day normal A/J serum (*solid squares*) or anti-I-J^k alloantiserum (*open squares*) and into immune (*open triangles*) or hyperimmune (*solid triangles*) A/J mice which had received one or several previous tumor challenges, respectively.

small satellite nodules were seen, and occasionally these were in the dermis. In a few animals most of the tumor was in the dermis. In some areas, especially in sites where there was leukocytic infiltration and necrosis, the tumor cells were isolated from one another, rather than present as syncytial masses. The neoplastic cells were slightly pleomorphic and there were numerous mitotic figures. As will be described, there was more necrosis in the immunized and anti-I-J^k antiserum treated animals than in the control animals. To a large extent this appeared to be associated with infiltration of the neoplasm by leukocytes; however, the amount of necrosis often seemed more extensive than could be accounted for on the basis of direct contact between neoplastic cells and leukocytes. Analysis of this aspect was difficult because of the occurrence of coagulative necrosis in the central part of tumor nodules in many animals, including controls (Figure 1); this change was probably the result of the tumor having outgrown its blood supply. There was mild to moderate fibroblastic proliferation, congestion, and increase in small blood vessels adjacent to the tumor nodules; these changes did not appear to vary among the groups or to increase appreciably with time.

The host cells seen in and around the tumor nodules included polymorphonuclear leukocytes (PMNs), mononuclear cells, and, less often, plasma cells. For purposes of description, the mononuclear cells were classified either as small mononuclear cells or macrophages. Most of the small mononuclear cells were probably lymphocytes, but some may have been

Table 1—Histologic Findings In Tumor (Experiment 1)

Day	Group (animal)	Necrosis: Central coagulative	Necrosis: Associated with leukocytic infiltration	Leukocytic accumulation: Adjacent to tumor	Leukocytic accumulation: Infiltrating tumor nodule	Leukocytic accumulation: Perivascular and perineural
Day 3	Control	+++	0	Trace	0	0
	Control	+	0	Trace	Trace	0
	IJ^k*	+	0	Trace	Trace	0
	IJ^k	+	+	+	+	0
	Immunized†	++	++	++	++	+
	Hyperimmunized	+	+	++	+	++
	Hyperimmunized	++	++	++	++	++
Day 7	Control	+++	Trace	+	+	0
	Control	+	++	+	++	0
	IJ^k	+++	+	++	+	+
	IJ^k	++	++	++	++	Trace
	Immunized	+++	++	+++	++	+++
	Hyperimmunized	No tumor present				
	Hyperimmunized	0	+++	+++	+++	++
Day 10	Control	+++	Trace	+	+	±
	Control	0	0	±	±	0
	IJ^k†	+++	++	+++	++	+++
	Immunized	++	+	+	+	0
	Immunized	+++	++	++	++	++
	Hyperimmunized	No tumor present				
	Hyperimmunized	++	++++	+++	+++	+++

* Batch 482 of 3R anti-5R used in this experiment
† One animal excluded due to unsatisfactory inoculation

monocytes. In general, the infiltrates adjacent to the tumors contained more mononuclear cells than PMNs (although PMNs were numerous in most cases), whereas PMNs often predominated within the tumor nodule. Although PMNs were frequently found in association with necrotic tumor cells, they were also found in sites where no necrosis was evident. In the vicinity of some tumor nodules, perivascular or perineural collections of small mononuclear cells or plasma cells were seen, sometimes as far as 200 to 300 μ from the tumor itself.

Experiment 1

The principle histologic features related to the tumor are presented in Table 1 and can be summarized as follows: At Day 3 in the control animals there were only a few leukocytes adjacent to the tumor and there was no necrosis associated with the infiltrating leukocytes (Figure 1). In the animals that had received anti-I-J^k serum, the leukocytic infiltrate was

slightly greater and in some areas leukocytes extended into the peripheral part of the neoplasm (Figure 2); in 1 animal there were foci of tumor necrosis associated with invading leukocytes. In the immunized and hyperimmunized animals there was appreciably more leukocytic infiltration and necrosis. In addition, perivenular and perineural collections of small mononuclear cells were found adjacent to the tumor.

At Day 7 the degree of leukocytic infiltration around the tumors in the control animals was somewhat greater than at Day 3, but it was still mild (Figure 3); irregular leukocytic invasion of the tumor with necrosis was seen. In the animals that had received anti-I-J^k serum, these changes were appreciably more marked (Figure 4), but, as at Day 3, they were not as severe as in the immunized or hyperimmunized animals. In the latter two groups the number of perivenular and perineural infiltrates had increased, and in 1 animal the collections contained numerous plasma cells.

At 10 days the degree of inflammation and necrosis in the nonimmunized animals continued to be mild (Figure 7). In contrast, in the anti-I-J^k treated animal there was intense irregular leukocytic infiltration within and around the tumor (Figure 8); in addition, numerous perivascular perineural collections of mononuclear cells were seen adjacent to the tumor (Figure 10). In the immunized animals these features were more marked. In the 1 hyperimmunized animal in which identifiable tumor tissue was present, there was almost complete destruction of the neoplastic cells; the area of the tumor was heavily infiltrated with leukocytes, most of which were PMNs. Adjacent to the tumor were prominent perivenular and perineural infiltrates which contained numerous plasma cells (Figure 11).

Spleen

There was moderate variation in the histologic appearance of the spleens in normal non-tumor-bearing controls (2 of which were killed on Days 3, 7, and 10) and in different animals in the same experimental group. However, the following changes appeared to exceed this kind of apparently random variation: On Day 3 the spleens in the immunized and, to a greater extent, in the hyperimmunized animals showed enlargement of the white pulp, increased germinal center activity, and increased numbers of blasts and plasmacytoid cells in the red pulp. At Day 7 these features were more marked in the immunized and hyperimmunized animals and were also present in the control and I-J^k treated mice, although to a lesser extent. By Day 10 the changes had subsided somewhat in all groups. There were no definite differences between the control tumor-bearing mice and the animals treated with I-J^k serum at any stage.

Lymph Nodes

At Day 3 the lymph nodes from the control tumor-bearing or anti-I-J^k treated mice showed slight increase in the paracortical areas. In the immunized and hyperimmunized group there was a greater degree of increase in paracortical areas; in addition, germinal centers were present and plasma cells were seen in the medullary cords.

At Day 7 the lymph nodes from the control tumor-bearing animals exhibited more pronounced paracortical hyperplasia but almost no germinal center formation; plasma cells were not seen. In the anti-I-J^k treated mice the extent of paracortical activity was even greater, but germinal center formation was minimal and plasma cells were not found. The lymph nodes from the immunized animals showed prominent germinal center formation, increase in paracortical zones, and plasma cells in medullary cords. These features were even more striking in the hyperimmunized group.

At Day 10 the hyperplastic changes had subsided somewhat in all groups. There were no histologic abnormalities in the livers or kidneys of any of the animals. No consistent changes were found in the thymus, although some exhibited depletion.

Experiment 2 (Tumor Day 8 Only)

The histologic findings in these animals (Table 2) can be summarized as follows: In all 4 control animals the tumor nodules were large and showed almost no necrosis. There was slight to moderate irregular leukocytic

Table 2—Histologic Findings In Tumor (Experiment 2)

Day	Group (animal)	Necrosis		Leukocytic accumulation		
		Central coagulative	Associated with leukocytic infiltration	Adjacent to tumor	Infiltrating tumor nodule	Perivascular and perineural
Day 8	Control	0	+	+	+	0
	Control	0	+	++	+	0
	Control	0	+	+	+	+
	Control	0	+	++	+	+
	I-J^k*	0	+++	+++	+++	+++
	I-J^k	+	++++	++++	+++	+++
	I-J^k	+	++++	++++	++++	++++
	I-J^k†	0	+++	+	+++	+

* Batch 480 of (3R × DBA/2)F$_1$ anti-5R used in this experiment
† Tumor almost completely destroyed

infiltration adjacent to the tumor and in the most peripheral portions of the neoplastic nodules (Figure 5). In most areas the infiltrate consisted predominantly of small mononuclear cells, but in some places PMNs were more numerous (Figure 9). There were very few perivascular or perineural collections of leukocytes adjacent to the tumor.

In contrast, in all 4 of the anti-I-J^k treated animals, the tumor showed intense leukocytic infiltration and necrosis (Figure 6). PMNs predominated within the tumor nodule, whereas in most areas around the tumor mononuclear cells were more numerous. Perivascular and perineural infiltrates were conspicious adjacent to the tumor. In 1 case, acute inflammation of the wall of a vein was seen. The effects of anti-I-J^k administration appeared more dramatic in this than in the previous experiment, possibly due to the batch of serum used with these animals.

Discussion

The S1509a methycholanthrene (MCA)-induced fibrosarcoma used in the present experiments has been extensively studied and has been shown to induce the generation of specific suppressor T cells which exert a dampening effect on the host reactivity to the tumor.[2,3] Recent work using MCA-induced sarcomas shows that anti-I-J alloantiserum administered *in vivo* decreases tumor growth and inhibits the development of tumor-specific suppressor T cells.[1] Such adjuvant effects could be the consequence of neutralization of suppressor T cells or their factors or, alternatively, but less likely, could be due to stimulation of another population of lymphoid cells.[13] We favor, however, the direct reduction of suppressor activity, since animals treated with anti-I-J antiserum can no longer adoptively transfer suppression, as has been previously shown.[1] We are aware, of course, that this reduction in suppressor cell function could be only relative to enhanced effector responses.

In the present study we have analyzed the effect of anti-I-J antiserum on the host response to the S1509a tumor by examining the histologic features of tumor and lymphoid organs of treated and control animals. We have compared these findings with those in animals which had been rendered immune to S1509a by previous challenge followed by surgical excision of the primary graft.

Our findings show that anti-I-J^k treatment results in an increased leukocytic reaction to the tumor as compared with controls. The reaction within and around tumor in the anti-I-J^k treated mice was characterized in most instances by an infiltrate in which small mononuclear cells predominated; neutrophils were also generally conspicuous and in some areas were more numerous than mononuclear cells. Perivenular and perineural collections

of mononuclear cells adjacent to the tumor were conspicuous in animals killed at 7, 8, or 10 days. Plasma cells were not found in the infiltrate. All of these features are consistent with, although by no means diagnostic of, a cell-mediated type reaction. The presence of numerous neutrophils does not constitute evidence against this interpretation, since these are frequently present in large numbers of delayed reactions, especially in the mouse. However, in some areas of the tumor, neutrophil accumulation appeared to be secondary to necrosis. Further evidence that the predominant response was cellular was provided by the changes in lymphoid tissue, with expansion of thymus-dependent zones without associated proliferation of plasma cells. Clearly, however, these findings do not exclude the participation of antibodies in the reaction against the tumor.

The histologic findings in the immunized and hyperimmunized animals were in most respects similar to although more severe than in the anti-I-J^k treated animals. However, in addition to the features mentioned above, plasma cell proliferation was seen in lymphoid tissue and collections of plasma cells were found adjacent to the tumors, indicating a humoral antibody response.

Although there was considerable leukocytic infiltration in the tumors undergoing necrosis, as has been observed in other tumor systems during immune rejection,[14] it did not appear that the amount of tumor destruction found in the experimental groups (as judged either grossly or microscopically) could be accounted for entirely on the basis of direct contact between leukocytes and neoplastic cells. Similar observations have been made in other experimental tumors undergoing rejection.[15] The present study provides no convincing evidence for other mechanisms of tumor destruction; however, the possibility that interference with the vascular supply of the tumor was involved is suggested by the finding of inflammation in and around the walls of vessels in some animals. Nevertheless, vascular occlusion was not demonstrated.

To the extent that leukocytes were directly responsible for damage to tumor cells, the histologic findings suggest that lymphocytes and neutrophils were the principal effector cells. Clearly identifiable macrophages were rarely seen in contact with tumor cells and were not especially conspicuous in the infiltrate adjacent to the tumor. The possible participation of mast cells or basophils was not evaluated in this study.

These results support the notion that reduction of suppressor cell influence by administration of anti-I-J alloantiserum potentiates the expression of effector cell activity *in vivo*. The histologic analysis of the tumors reveals similarities between antiserum-treated nonimmune animals and their immune counterparts in the type of cellular response associated with

tumor elimination. It can only be inferred from these studies, however, that the enhanced cellular response of nonimmune animals is a direct result of the abrogation of a suppressive influence. Other lines of evidence which appear to substantiate this interpretation include the effects of antithymocyte serum (ATS), which, when administered *in vivo* at defined times following tumor inoculation, eliminates suppressor T-cell activity and similarly leads to limited tumor growth.[3] Abrogation of suppressor cell activity by the use of adult thymectomy [16] or cyclophosphamide therapy [17] results in restricted tumor growth.

Although therapy with anti-I-J antiserum has repeatedly been shown to cause a reduction in development and rate of appearance of tumor, complete tumor elimination has not been achieved by this treatment alone. The failure to eradicate tumor was reflected in the present study by the finding that the cellular response of treated nonimmune animals to the tumor is consistently less dramatic than in the immune or hyperimmune host. Thus, reduction of suppressor cell function alone is insufficient to allow generation of the degree of responsiveness required to eliminate a rapidly growing tumor mass in the case of sarcoma S1509a. Experiments designed to increase the effectiveness of anti-I-J therapy by coupling these treatments with other modes of immunotherapy are in progress. By such combinations of therapy, it may be possible to specifically shift the balance of immune reactivity in favor of the primary tumor-bearing host by simultaneously reducing suppressor cell influence and augmenting effector reactivity.

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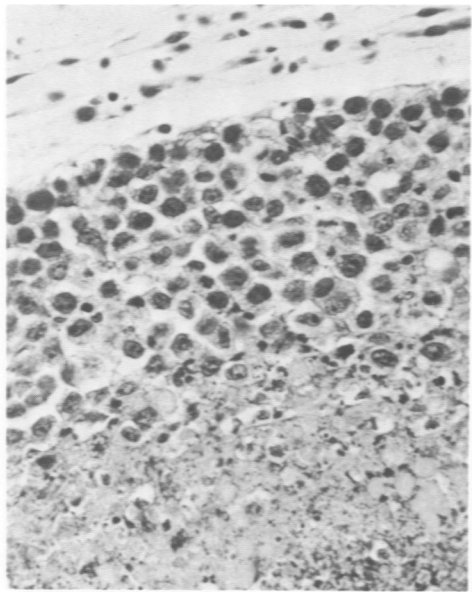
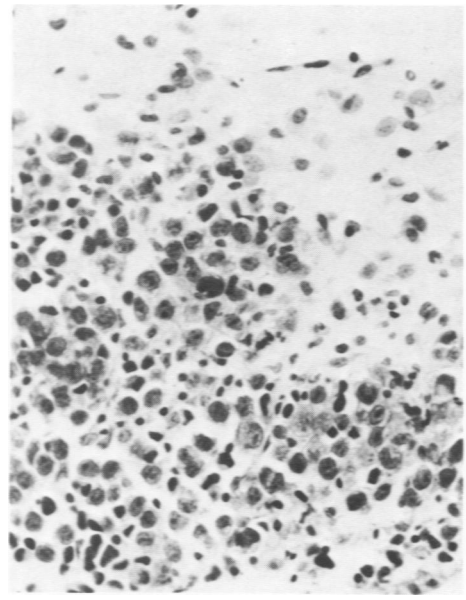
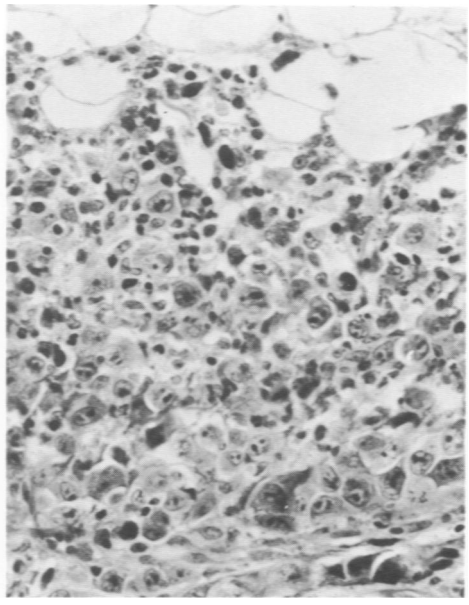
[*Illustrations follow*]







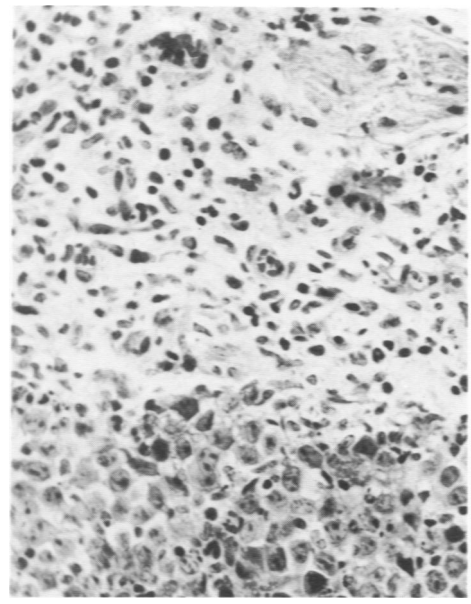


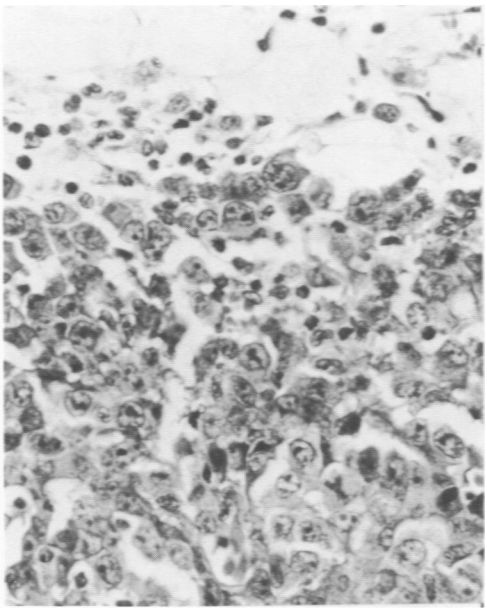
Figure 1—Tumor from a control animal at Day 3. Only a few leukocytes are seen adjacent to the tumors. There is coagulative necrosis of the central part of the tumor nodule. (H&E, × 290) **Figure 2**—Tumor from anti-I-J^k treated animal at Day 3. The tumor is infiltrated by moderate numbers of small mononuclear cells. (H&E, × 290) **Figure 3**—Tumor from control animals at Day 7. The peripheral portion of the tumor is infiltrated by small numbers of mononuclear cells. (H&E, × 320) **Figure 4**—Tumor from anti-I-J^k treated animal at Day 7. There is a moderate mononuclear infiltrate adjacent to and within the tumor. Many of the neoplastic cells are damaged or destroyed. (H&E, × 320)

Figure 5—Tumor from control animal at Day 8. There is slight mononuclear cell infiltration of the peripheral part of the tumor, without necrosis. (H&E, × 320)

Figure 6—Tumor from anti-I-J^k treated animal at Day 8. An intense leukocytic infiltrate is seen adjacent to and within the peripheral portion of the tumor nodule. Many of the tumor cells appear damaged. Most of the leukocytes are small mononuclear cells. (H&E, × 350)

Figure 7—Tumor from control animal at Day 10. Only mild leukocytic infiltration of the neoplasm is present. (H&E, × 315)

Figure 8—Tumors from anti-I-J^k treated animal at Day 10. There is intense leukocytic accumulation adjacent to and within the neoplasm. Many of the tumor cells have been destroyed. (H&E, × 315)



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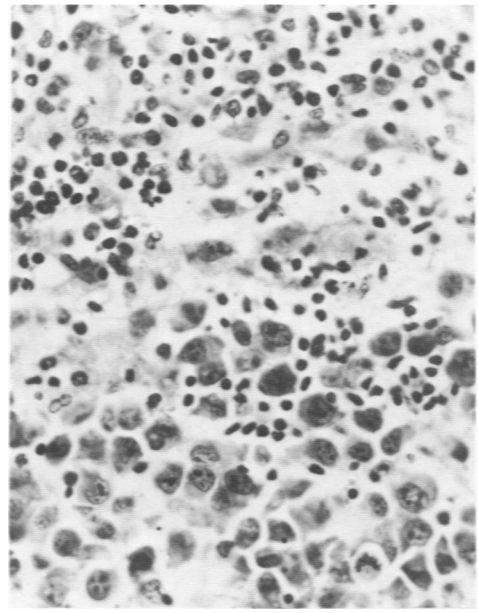

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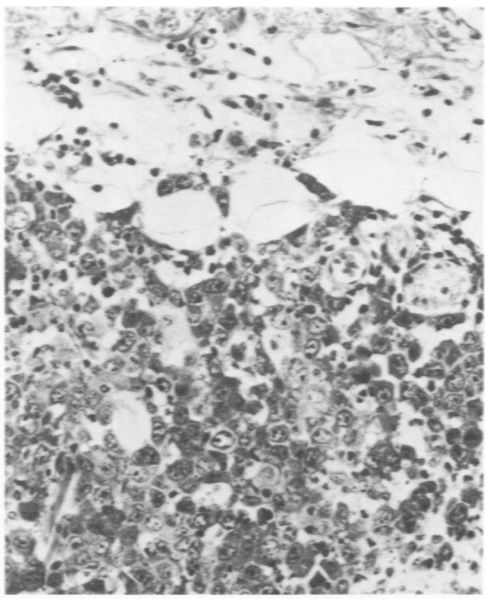

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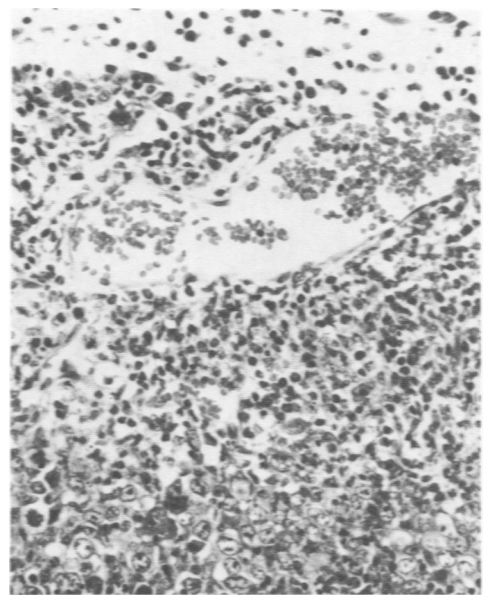

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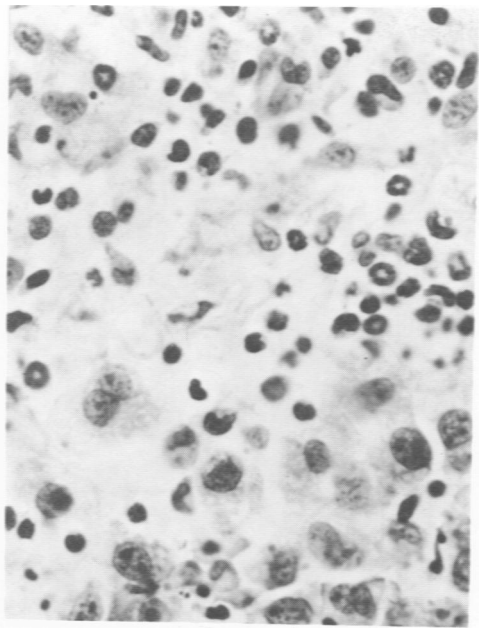

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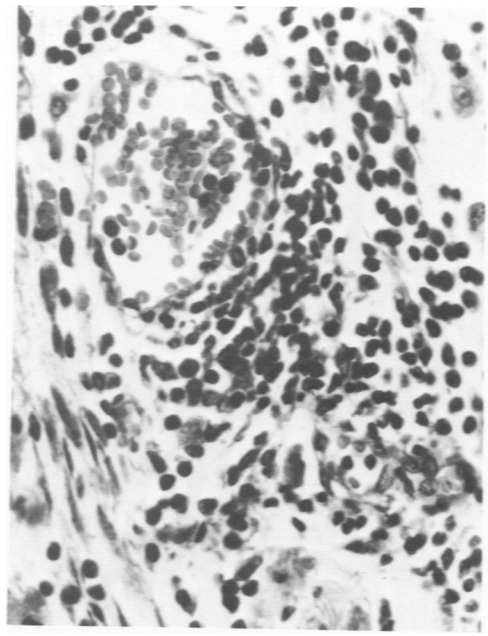

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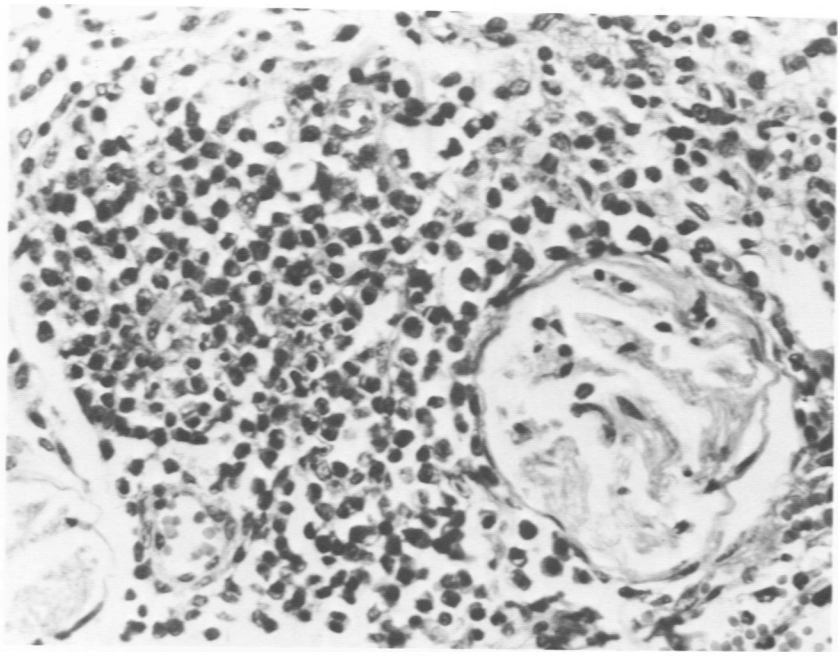

Figure 9—An area of tumor from an anti-I-J^k treated animal at Day 8. The tumor is heavily infiltrated with leukocytes, many of which are neutrophils. (H&E, × 600) **Figure 10**—An area adjacent to the tumor from an anti-I-J^k treated animal at Day 10. There is accumulation of small mononuclear cells adjacent to a venule. (H&E, × 410) **Figure 11**—An area adjacent to the tumor from a hyperimmunized animal at Day 10. Numerous plasma cells are seen adjacent to a small nerve. (H&E, × 410)