

Kinetics of the Plasmacellular Reaction in the Draining Lymph Node of Mice Bearing the BP-8 Fibrosarcoma

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SUMMARY

The kinetics of the plasmacellular response in the draining lymph node was quantitatively assessed in specific-pathogen-free C3H/He mice grafted s.c. with the syngeneic BP-8 fibrosarcoma. The percentage of plasma cells was estimated on dissociated, cytocentrifuged, and fixed cell preparations by immunoenzymatic staining with either of two B-cell-specific antibody markers. Mice either grafted with live tumor or given a single implant of tumor homogenate gave a maximal plasma cell response of about 4%. However, this peak was reached at 14 days in the live grafts and at 7 days in the homogenate situations. Moreover, in animals grafted with live tumor an elevated 3% plateau persisted until death, whereas in homogenate-implanted animals a sharp drop to a 0.5% plateau was observed at about Day 20. Homogenate-treated animals displayed a significant enhancement of tumor growth when challenged with a live graft 7 days after homogenate implantation, *i.e.*, during the peak 4% plasmacellular response. Mice given a single implant of X-irradiated (3500 R) tumor responded with a peak of 1.7% plasma cells at 7 days. Despite subsequent boosting with the same material (Days 14 and 21), this value declined and remained at a plateau of about 0.2% until death. Contrary to homogenate-treated mice, these animals were immune to subsequent challenge with a live tumor graft. In the present host-tumor system, there appears to be an inverse relationship between elevated numbers of plasma cells and antitumor immunity.

INTRODUCTION

It is now well established that the s.c. grafting of tumor induces an intense immune reaction in the host's draining lymph nodes. Why this reaction fails to protect the host and how the cellular events in the lymph node relate to this have been the subject of much effort by investigators. Nevertheless, it has been shown that a relative degree of acquired immunity is definitely associated with the regional lymph node. Thus, Flannery *et al.* (14) have shown that the regional lymph node is the site that reacts earliest to the implantation of a syngeneic tumor. Also, in a syngeneic situation, Fisher and Fisher (11) have shown that the surgical removal of regional nodes prior to tumor implantation resulted in a distinctly lower level of immunity compared with that of animals in which nodes were left *in situ*. More-

over, the prevailing view is that antitumor immunity is associated with cell-mediated immune mechanisms (5, 6, 12, 17, 19) rather than humoral antibodies (8) and that the latter may in certain cases interfere with the former through serum "blocking factors" (4, 16, 20).

The significance of the cellular events in the regional lymph node of tumor-bearing animals has been intensely studied, the aim being mainly to establish whether a particular histological feature of such reacting nodes had specific bearing on the outcome of a live graft situation. The original observations of Parsons (21) have now been confirmed (7, 9, 13). Some of the outstanding features of such nodes are the pronounced immunoblastic and plasmacellular responses associated with lymph node hyperplasia, and parallels have been drawn between situations of skin grafting and conventional immune responses.

Whatever the biological significance of the cellular events in the lymph node, we believe that an approach based on the quantitative assessment of their evolution should provide a more critical basis for appraising their role. This work was restricted to the study of 1 cell lineage of the lymph node, *i.e.*, the plasma cell, in mice grafted with the BP-8 fibrosarcoma. The study was performed using B-cell-specific peroxidase-conjugated antibodies on preparations of dissociated and cytocentrifuged lymph node cells. An attempt was made to distinguish between the patterns of the plasmacellular response occurring during progressive growth of a live graft *versus* that occurring following an implant of either X-irradiated tumor or of disrupted tumor cells.

MATERIALS AND METHODS

Animals. Six- to 8-week-old inbred C3H/He female mice were used. They were bred and experimented with in a specific-pathogen-free colony at our institute's animal house.

The BP-8 Fibrosarcoma.¹ This tumor was kindly donated by Dr. I. Chouroulinkov. The tumor was maintained in the solid form in C3H/He mice by serial s.c. passage in the back region. It causes 100% mortality 40 to 60 days postgrafting. During the course of these experiments, a routine examina-

¹ Because many transplant generations have elapsed since this tumor had been originally induced in C3H mice, there may have occurred a change in the H-2 makeup of the tumor. Furthermore, histocompatibility antigens in the mice themselves may have changed or been lost over the years as a result of genetic drift. In the latter case, this would allow recognition of the H-2 of the tumor antigens by the present-day hosts as "neoantigens."

Received December 2, 1975; accepted April 30, 1976.

tion performed by Dr. J-C. Guillon of the Pasteur Institute, Paris, France, showed that the tumor was free of bacteria as well as the choriomeningitis and rheoviruses. For challenge, a 2- to 3-mm fragment was implanted s.c. in the flank proximal to the axillary node.

Tumor homogenate was prepared by 3 consecutive freeze-thaw cycles of 19-day-old excised grafts. The tumors were then processed in a Potter vessel in one-third volume of ice-cold unsupplemented Eagle's minimal essential medium. Each mouse received a single implant of 0.15 g tumor (wet weight) administered in the axillary region.

X-irradiated tumor (3500 R) was prepared from minced 20-day-old excised tumor grafts. Twenty-two animals received a total of 3 s.c. grafts of 0.15 g tumor (wet weight) in the axillary region at Days 0, 14, and 21. Ten mice of this group were challenged with live BP-8 tumor on Day 28 in the same region.

Preparation of B-cell-specific Antibodies. Two distinctly different reagents were used. One was Ra-kappa² antibodies, the other was Ga-Mic antibodies. The Ra-kappa was obtained by immunizing with the Fab fragment of an Ig G2a, κ myeloma globulin and by adsorbing the serum on an immunoadsorbent of the Fc fragment of the same myeloma protein. The anti- κ antibodies were then isolated from the specific anti-K serum by adsorption and elution from an Ig G1, κ myeloma globulin immunoadsorbent (3). The Ga-Mic was obtained by immunizing with BALB/c myeloma microsomes and then by exhaustively adsorbing with serum, BALB/c liver, and erythrocyte immunoadsorbents. The antibodies were isolated from the resultant serum by adsorption and elution from a myeloma microsome immunoadsorbent. The technical aspects of these procedures have been described in full detail elsewhere (24, 25). Antibodies were conjugated with horseradish peroxidase according to the method of Avrameas (2).

Labeling of Lymph Node Plasma Cells. Both reagents react with the intracellular antigens of plasma cells. The Ra-kappa antibodies combine with the immunoglobulin light κ chains borne in mice by 95 to 97% of serum immunoglobulins (18) and presumably by a similar proportion of plasma cells. The Ga-Mic antibodies react with cytoplasmic plasma cell antigens shown to be distinct from immunoglobulin determinants (M. Stanislawski, unpublished data). At the various time points following grafting or implantation indicated in "Results," 3 mice were sacrificed and their axillary and brachial nodes, draining the inoculated site, were removed and placed singly or pooled (see "Results") in the ice-cold isotonic medium of Shortman *et al.* (23) supplemented with 2% bovine serum albumin (Nutritional Biochemicals Corp.; Fraction V). The cells were released into the medium by gentle teasing with sharp forceps and were washed twice in the same medium by centrifuging at 1500 $\times g$ for 7 min in a refrigerated centrifuge. Cell viability, as determined by trypan blue exclusion, ranged from 85 to 95%. The cells were immediately cytocentrifuged on microscope slides, using a Shandon-Elliott cytocentrifuge, at

1000 rpm for 5 min, dried, fixed, and stained with antibodies according to Method D, details of which have been reported (26).

For each determination, 1 or 2 slides were labeled with Ra-kappa and the same number with Ga-Mic at the following antibody concentrations and incubation times: Ra-kappa (50 $\mu\text{g/ml}$) for 30 min, followed by Sa-R IgG-Po (250 $\mu\text{g/ml}$) for 90 min; Ga-Mic (90 $\mu\text{g/ml}$) for 30 min, followed by Ra-G IgG-Po (250 $\mu\text{g/ml}$) for 90 min. 3-Amino-9-ethylcarbazole (15) was used as the colored reaction product in order to reveal peroxidase activity.

Specificity of staining was controlled as follows. For endogenous peroxidase activity, untreated, fixed slides were flooded in 3-amino-9-ethylcarbazole solution. No cells, including the rare erythrocytes and cells of the granulocytic series present in lymph node cell suspensions, were stained. Antibody specificity was controlled by treating fixed slides with peroxidase-conjugated normal rabbit IgG (500 $\mu\text{g/ml}$) or normal rabbit IgG (75 $\mu\text{g/ml}$) followed by Sa-R IgG-Po (250 $\mu\text{g/ml}$), or only the Sa-R IgG-Po (250 $\mu\text{g/ml}$) or only Ra-G IgG-Po (250 $\mu\text{g/ml}$). To control further the specificity of the antibodies used, a sample of Ra-kappa was preincubated with normal mouse serum and then applied on slides, followed after 30 min by Sa-R IgG-Po. Similarly, Ga-Mic was adsorbed with live myeloma or spleen cells and applied on slides. In both cases plasma cell staining was nonexistent.

Plasma cells, which were deeply pink, were counted using an ordinary light microscope equipped with a vernier scale. The cell spot was screened at $\times 250$, and positive plasma cells were scored and expressed as the percentage of overall nucleated cells per slide.

RESULTS

Kinetics of the Plasmacellular Response following a Graft of Live BP-8 Tumor. As shown in Chart 1, there was a sharp increase of the plasma cells soon after tumor implantation; this reached a peak of 4% at 14 days. This response then slightly abated but remained at a relatively high level of 3% until sacrifice at 40 days.

Slides labeled with either Ra-kappa or Ga-Mic gave very similar percentage values, indicating that either reagent was convenient for plasma cell estimation. There was 1 exception at 4 days. Here, while no plasma cells could be detected with Ra-kappa, the Ga-Mic reagent gave a value of 0.5%. Careful examination of these slides revealed that the cells labeled with the latter reagent, although displaying characteristic plasma cell morphological features, resemble the more immature plasma cell types. These cells apparently synthesized insufficient cytoplasmic immunoglobulin to be visualized with Ra-kappa. However, since no statistically significant difference was found between these reagents, the Ga-Mic was used in subsequent experiments.

Kinetics of the Plasmacellular Response following a Single Implant of BP-8 Tumor Homogenate. This experiment was an attempt to assess the relative capacity of the disrupted tumor, containing cell surface and hypothetically released intracellular antigens, to mount a plasmacellular response in the regional lymph nodes. None of the mice given 0.15 g of this material developed tumors as judged by

² The abbreviations used are: Ra-kappa, rabbit anti-mouse κ -chain antibodies; Ga-Mic, goat anti-mouse myeloma microsome antibodies; Sa-R IgG-Po, sheep anti-rabbit immunoglobulin G antibodies conjugated with horseradish peroxidase; Ra-G IgG-Po, rabbit anti-goat immunoglobulin G antibodies conjugated with peroxidase.

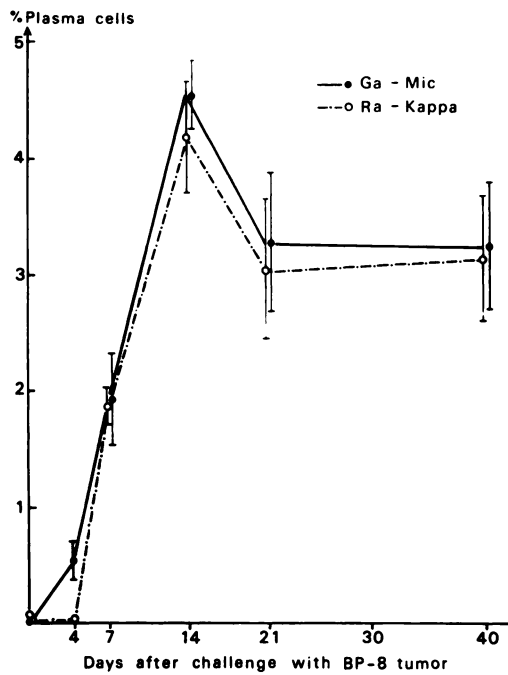


Chart 1. The draining lymph node plasmacellular response of mice grafted s.c. with the BP-8 fibrosarcoma at Day 0. The plasma cells were estimated on dissociated, cytocentrifuged, and fixed cell suspensions as described in "Materials and Methods," using either Ra-kappa or Ga-Mic antibodies. The data were compiled from 3 separate experiments. Points, mean \pm S.E. of axillary and brachial nodes from 3 mice that were pooled or analyzed singly. Except at Day 4, there was no statistically significant difference between the Ra-kappa and Ga-Mic values.

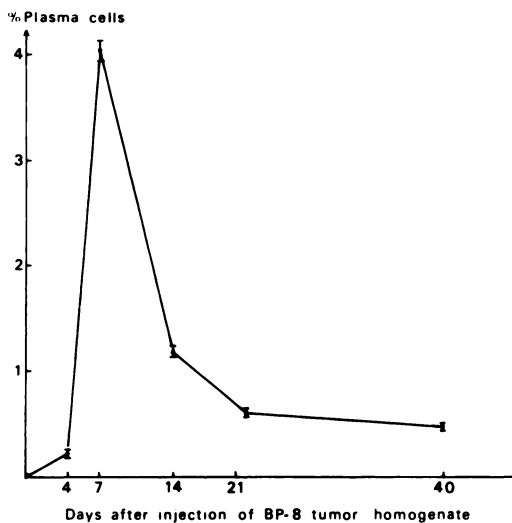


Chart 2. The draining lymph node plasmacellular response of mice implanted on Day 0 with 0.15 g (wet weight) of BP-8 tumor homogenate. The plasma cells were estimated with Ga-Mic antibodies. Mean \pm S.E. of singly analyzed axillary and brachial nodes from 3 mice.

surgical opening of the implanted site at sacrifice. As seen in Chart 2, the response obtained, although as intense as that obtained with a live tumor graft, differs qualitatively from the latter. Again a 4% peak response was obtained; however, this was seen earlier (7 days) than in the live graft situation. Subsequently, the plasma cells declined rather sharply and remained at a 0.5% plateau until 40 days.

In another experiment, we studied the influence of homogenate pretreatment of mice on the subsequent tumor

development upon challenge with a live graft. As shown in Chart 3, tumor development was significantly enhanced when mice were challenged 7 days after homogenate implantation. This is the time when the plasmacellular response is at its peak intensity (see Chart 2). However, when mice were challenged at 14 days after homogenate injection, a time when the plasma cells had declined to about 1%, such an enhancing effect was not observed.

Kinetics of the Plasmacellular Response following Grafting with X-irradiated BP-8 Tumor. This experiment was an attempt to determine whether an X-irradiated mitotically inactive tumor graft can mount a plasmacellular response.

A similar amount of tumor as in the homogenate experiment was administered at Day 0, and the number of implants and the schedule of their administration that was adopted were those that established a definite state of immunity in preliminary experiments. Of 10 mice thus treated that were subsequently challenged with a live tumor graft, none developed tumors 30 days later, while 5 of 5 untreated controls that received a similar graft at the same time developed tumors and died within 40 to 60 days.

As shown in Chart 4, the pattern of the plasmacellular response is similar to that obtained with tumor homogenate. The major difference is that, here at the 7-day peak response, the plasma cell count was only 1.7% instead of the 4% obtained with homogenate. The plasma cells then declined and remained at a low 0.2% plateau until sacrifice at 40 days. Reimplantation of X-irradiated tumor in the same region at Days 14 and 21, as well as grafting with live tumor at Day 28, did not significantly alter the percentage of plasma cells.

DISCUSSION

The peripheral lymph nodes of untreated C3H/He mice bred in specific pathogen-free conditions contained <0.01% plasma cells. This is in contrast to, for example, the spleen of these animals in which counts of 5 to 7% are regularly encountered (M. Stanislawski, unpublished data). The peripheral lymph node is therefore particularly well suited for the quantitative studies described here, since the bulk of its plasmacellular response may be attributed as a reaction to the antigens of the tumor.

Previous histological studies of the cellular reactions occurring in lymph nodes of tumor-bearing mice have emphasized the intense plasmacellular proliferation as being a prominent feature of such nodes (7, 9, 13, 22). Because these studies were performed using conventionally stained tissue sections, a truly quantitative appraisal of these cells was difficult to make. In view of recent reports concerning interference between the cell-mediated and humoral compartments in antitumor immunity (4, 16, 20), it seemed to us particularly warranted to approach the question of plasmacellular proliferation using a quantitative approach. The method adopted was to identify specifically the plasma cells in fixed, single-cell preparations of reacting regional lymph nodes using peroxidase-conjugated antibodies directed either to cytoplasmic immunoglobulin or to a plasma cell xenoantigen. Such an approach yielded reproducible esti-

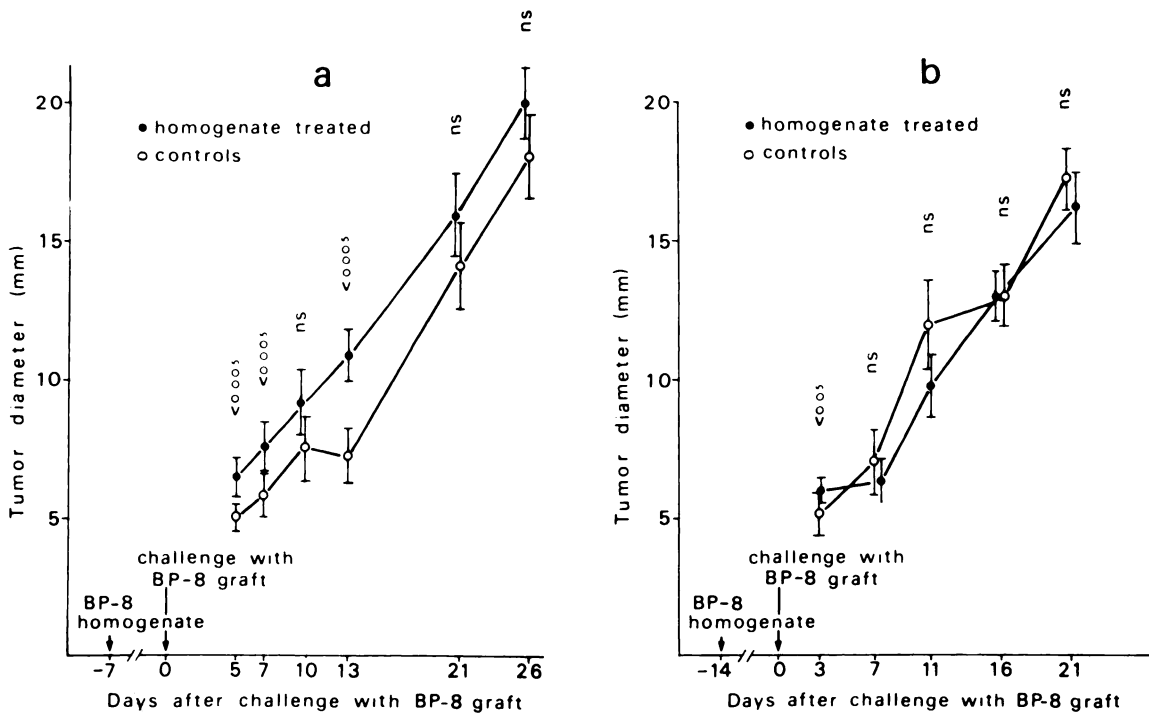


Chart 3. Tumor development in BP-8 homogenate-treated mice. Ten mice were implanted with BP-8 homogenate in the right axillary region, and 6 of these were challenged in the same site with a live tumor graft 7 days later (a), whereas the other 4 were similarly challenged 14 days later (b). Control mice were challenged simultaneously, 5 mice in a and 7 mice in b. Points, mean \pm S.E. Vertical numbers, homogenate treated versus controls. ns, not significant.

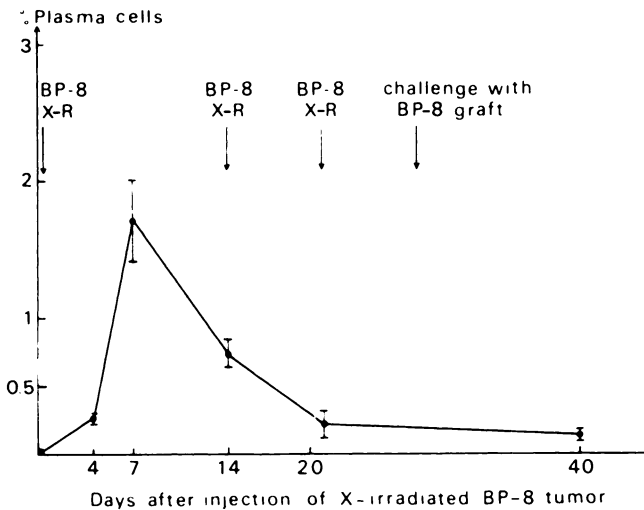


Chart 4. The draining lymph node plasmacellular response of mice implanted with X-irradiated BP-8 tumor. Tumor (0.15 g. wet weight) was given on Days 0, 14, and 21, and the mice were challenged on Day 28 with a live graft. The plasma cells were estimated with Ga-Mic antibodies. Points, mean \pm S.E. of singly analyzed axillary and brachial nodes of 3 mice.

mates of the kinetics of this cell type in different situations of tumor implantation. Thus, in order to evaluate more knowingly the type and extent of plasmacellular proliferation taking place during progressive tumor growth, it was desirable to compare this with the kinetics of proliferation in situations where either resistance to challenge or enhancement are obtained. Such biologically different host responses were created by the inoculation of either X-irradiated tumor or disrupted tumor cells.

From the overall results obtained, but with the reservation that the study was performed in only 1 host-tumor system,

an inverse correlation is seen between the intensity and duration of the plasmacellular infiltration of the draining lymph node and the immune status of the animal. Elevated numbers of these cells are found in situations where immunity is lacking, *i.e.*, during tumor growth (Chart 1) and homogenate implantation (Chart 2). On the other hand, low numbers are present in 1 situation where tumor resistance is induced, as in the X-irradiated tumor experiment. Here, the 1.7% peak response is a relatively moderate response compared with the other 2 groups and, at any rate, is the lowest obtained (compare Chart 4 with Charts 1 and 2). This response is not necessarily incompatible with the above-mentioned correlate since the triggering of the cell-mediated and humoral immune compartments may be coincident, as has been discussed by Edwards *et al.* (9). More significantly, this correlation does hold at a time when the animals are challenged with a live tumor graft. Here resistance is displayed when the plasma cells are at a low of 0.2%.

Significant enhancement was obtained in the homogenate-implanted group at a time when the plasma cells were at their peak value (about 4%) (Chart 2). This capacity to enhance a tumor graft also correlated with the intensity of the plasmacellular response, since the effect was no longer observed in animals challenged at 14 days, a time when the cells had declined to about 1% (Chart 2). Since the plasma cell is the cellular entity that synthesizes and secretes most of the bodies' antibodies (10), these results are directly comparable to a previous report of enhancement in tumor transplantation immunity, which demonstrated that the phenomenon can be mediated by the passive administration of serum antibodies (1). The characteristic kinetics of plasmacellular proliferation seen in the live graft experi-

ments, particularly the persistence of a 3% plateau of these cells implying the secretion of rather large amounts of anti-tumor antibodies, is compatible with the data of Baldwin *et al.* (4) that an *in vitro* blockade of antitumor cell-mediated cytotoxicity is mediated by antigen-antibody complexes.

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