Immunobiology of primary intracranial tumors

Part 4: Levamisole as an immune stimulant in patients and in the ASV glioma model

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Levamisole was evaluated as an immune stimulant in a randomized controlled study of patients with anaplastic gliomas, who had undergone surgical resection and who were also treated with radiotherapy and BCNU chemotherapy. Of 102 patients placed into the study, 85 were determined to comprise the adequately treated group (ATG): a full course of radiotherapy and two cycles of BCNU chemotherapy. Within the ATG, those patients who received levamisole did not demonstrate significantly different serial delayed hypersensitivity reactions, peripheral blood lymphocyte and T-cell counts, or serum IgM levels, compared to those patients not receiving levamisole. There was no significant difference in survival times of the two groups. Studies utilizing the avian sarcoma virus-induced glioma in rats also showed no improvement in survival with levamisole stimulation as the only immune agent, but the combination of active immunization and adjuvant stimulation with bacillus Calmette-Guerin plus levamisole was found to be therapeutically effective in this model and will be used in future pilot studies of active immunization in patients.

Key Words • levamisole immunostimulation • malignant glioma therapy • glioma model • radiotherapy • BCNU chemotherapy

For the 7700 patients diagnosed each year as having malignant gliomas, and for those of us who treat them, the satisfactory management of this disease remains an enigma. Malignant gliomas are invariably fatal, with a median survival time after surgery alone of approximately 17 weeks. To date, the most effective treatment is a combination of surgical resection, whole-brain radiotherapy, and systemic chemotherapy with BCNU (1,3-bis-chloroethyl-1-nitrosourea). This treatment protocol, however, only increases median survival time to 62 weeks after surgery. Therefore, clinical trials of other agents, therapeutic modalities, and combinations of therapies are indicated.

We have previously reported evidence that patients with malignant gliomas have impaired host immunocompetence. This paper reports our experience with the adjunctive use of levamisole (2,3,5,6-tetrahydro-5-phenylimidazo-2,1-b-thiazole hydrochloride) as an immune stimulant in a randomized, controlled clinical trial in patients with malignant gliomas, also being treated with radiotherapy and BCNU chemotherapy. Correlative studies involving a variety of immunotherapy trials in the avian sarcoma virus (ASV)-induced glioma animal model also will be described.

Materials and Methods

Clinical Trials

Patients eligible for this study were identified within 3 weeks of subtotal surgical resection of a histologically proven supratentorial malignant glioma. None of these patients was receiving steroids at the time of entry into the study. After giving informed consent, patients underwent postoperative computerized tomography (CT) brain scanning, baseline neurological examination, Karnofsky functional rating, immunological screening, and hematological studies.

Host immunocompetence was determined by: 1) peripheral blood lymphocyte count with T-cell quantitation, 2) serum immunoglobulin quantitation, and 3) intradermal skin testing with common recall antigens: purified protein derivative (PPD), mumps,
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trichophyton, and streptokinase-streptodornase (SK/SD). In vitro microcytotoxicity assays in an autologous system were performed on four patients, utilizing the methods described previously. These assays are designed to detect relatively specific antigioma cellular, humoral, and/or antibody-dependent cellular cytotoxicity (ADCC). Hematological studies included hematocrit, leukocyte count, platelet count, alkaline phosphatase, bilirubin, creatinine, and blood urea nitrogen (BUN). Each patient was then randomly selected for treatment with levamisole or not. Those patients randomized to levamisole immunostimulation received the drug orally, 2.5 mg/kg, 3 days a week, every other week. Levamisole was discontinued if the patient developed a rash, persistent fever, or significant anorexia and weight loss. All patients were treated with radiotherapy (cobalt source, 200 rads/day, 5 days/week, whole-head therapy, with bilateral opposing ports, to a total dose of 4500 to 6000 rads over 5 to 6 weeks) and BCNU chemotherapy (80 mg/sq m/day intravenously for 3 days, repeated every 8 weeks). Chemotherapy and radiotherapy (with or without levamisole immunostimulation) were begun within 3 weeks after surgery, with the chemotherapy being repeated in 8-week cycles. At the beginning of each chemotherapy cycle, each patient was reevaluated by neurological examination, Karnofsky rating, immunological studies, chest x-ray film, and hematological studies, with CT brain scan optional. The BCNU dose was reduced to 60 mg/sq m/day if the previous cycle had caused a decrease in platelet count to less than 50,000 cells/mm, or a decrease in leukocyte count to less than 2000 cells/mm. Patients were followed until death, and their survival was analyzed by actuarial methods, using the Peto log-rank program to derive confidence intervals at 2 years and to compare the overall curves.

ASV Model Studies

Five-day-old Fischer 344 (CDF) rats were inoculated intracerebrally by freehand percutaneous administration of 2 µl of ASV suspension. The ASV suspension was a 1500-fold concentrate of tissue culture supernate. It was stored at -70°C in 0.05 mM sodium citrate buffer until use, when it was quickly thawed to 37°C. Injections were made via the open coronal suture into the right cerebral hemisphere using a ½-in., No. 30 needle, designed to place the inoculum near the lateral ventricular wall. The rats were randomly assigned to foster mothers. When 30 days old, the young rats were weaned and randomized into experimental groups of 20 rats each with equal numbers of males and females. A set of virus-inoculated rats, not included in the experimental groups, was killed at the time of randomization, and paraffin sections of their brains were stained with hematoxylin and eosin and examined to confirm the presence of brain tumors. In all cases, microscopic evidence of brain tumors was obtained.

The experimental rat study groups related to the clinical levamisole study as follows: 1) controls (no treatment); 2) radiotherapy plus BCNU chemotherapy; and 3) radiotherapy, BCNU chemotherapy, plus levamisole. Additional immunotherapeutic trials employing this animal model consisted of: 1) controls (no treatment); 2) bacillus Calmette-Guerin (BCG); 3) sarcoma cells (live); 4) levamisole; 5) BCG plus sarcoma cells (live); 6) BCG plus sarcoma cells (killed); 7) BCG plus sarcoma cells (live) plus levamisole; and 8) BCG plus sarcoma cells (live) plus levamisole plus BCNU chemotherapy. These experiments were performed to provide insight into future clinical trials. After randomization, two rats were housed in a cage with water and rat chow ad libitum. Treatments were begun 7 days after randomization (age 37 days). Rats were followed until death, at which time necropsies were performed and the brains examined grossly and histologically. A comparison of the survival time for each group was plotted, and the results analyzed by Wilcoxon rank sum statistical analysis.

Frozen BCG* suspension, containing 2-6 × 10⁸ organisms/ml, was stored at -70°C until used, when it was quickly thawed to 37°C. The S-262 clone 2 cell lines, established in tissue culture from a soft-tissue sarcoma induced by the ASV in BD-IX rats, served as the source of histo-incompatible tumor cells for active immunization. These sarcoma cells accordingly undergo immunological rejection after intraperitoneal injection and do not grow as separate tumors. However, the virus-determined cell surface transplantation antigens are presented to the host rats bearing ASV-induced brain tumors. The sarcoma cells were harvested from tissue culture and suspended in medium without fetal calf serum at a concentration of 2 × 10⁷ cells/ml. When nonviable cells were required, the sarcoma cells were killed by irradiating 50 ml of cell suspension in a plastic tube of 10,000 rads, given over 14.78 minutes with a cobalt-60 machine at a target-source distance of 40 cm and using no filtration. Death of irradiated cells was confirmed by failure of these cells to grow in tissue culture. All injections of BCG and/or sarcoma cells were given intraperitoneally. Sarcoma cells and BCG were mixed together in a ratio of one part of BCG to five parts of cell suspension, and a dose of 0.6 ml of this mixture (2-6 × 10⁷ BCG organisms and 10⁷ sarcoma cells) was given only once to each rat.

Each rat receiving BCNU, dissolved in an ethanol-water solution, was injected intraperitoneally with a single dose of 10 mg/kg of body weight. Levamisole was dissolved in sterile water and injected intraperitoneally in a dose of 2.5 mg/kg body weight twice weekly (Tuesday and Thursday) until the rats' death.

*Phipps strain BCG from the Trudeau Institute Mycobacterial Culture Collection was kindly supplied by Dr. Herbert J. Rapp of the National Cancer Institute.
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Results

Clinical Trial

One hundred and two adult patients constituted the valid study group (VSG). The VSG comprised all randomized patients with histologically proven anaplastic glioma who were available for follow-up evaluations. The adequately treated group (ATG) consisted of those patients who received a full course of radiotherapy and at least two cycles of BCNU chemotherapy. The ATG comprised 85 patients, of whom 57 had glioblastoma multiforme and 28 had other anaplastic gliomas. Forty-five patients of the ATG received levamisole and 40 did not. At the time of this writing, 21 patients are still alive in the ATG, of whom 12 are receiving levamisole and nine are not. Median survival time to date for those patients receiving levamisole is 348 days as compared to 353 days for those not receiving levamisole. The survival curves for all patients in the ATG (Fig. 1) reveal that there is no difference for the two groups of patients (p = 0.4767). When the effects of levamisole immunostimulation were assessed separately for patients with glioblastoma multiforme or other anaplastic gliomas, again there was no significant difference in median survival time of patients receiving levamisole and those who did not.

![Fig. 1. Life table showing the probability of survival versus survival time (in days) for all patients in the adequately treated group of patients with anaplastic gliomas.](image)

![Fig. 2. Life table showing the probability of survival versus survival time (in days) for levamisole-treated patients with glioblastoma, and with other anaplastic gliomas.](image)

![Fig. 3. Scattergram of serial delayed hypersensitivity reactions for patients with glioblastoma who received (black circle) or did not receive (white circle) levamisole. Each circle represents one patient.](image)

![Fig. 4. Scattergram of serial peripheral blood lymphocyte counts for patients with glioblastoma who received (black circle) or did not receive (white circle) levamisole. Each circle represents one patient.](image)
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Patients with glioblastoma multiforme had shorter median survival times than those with other anaplastic gliomas (Fig. 2). The median survival time for the levamisole-treated patients with glioblastoma multiforme is 275 days versus 775 days for levamisole-treated patients with other anaplastic gliomas.

Sequential determination of immunocompetence has been analyzed for all patients receiving or not receiving levamisole. Figures 3, 4, 5, and 6 show these data with reference to skin test delayed hypersensitivity reactions (DHR's), peripheral blood lymphocyte counts, percentage of T-lymphocytes in the peripheral blood, and serum IgM levels, respectively, for those patients with glioblastoma multiforme who have had sufficient serial studies done. Data from patients with other anaplastic gliomas were excluded from these figures because our previous studies have indicated general immune responses nearer normal in those patients as compared to those of patients with glioblastoma multiforme. The baseline immune responses of patients with glioblastoma multiforme randomized to receive levamisole were not significantly different by analysis of variance from those not receiving levamisole. Furthermore, there did not appear to be any significant maintenance or alteration of serial immune responses over an 8-month period. In the few patients receiving levamisole evaluated serially with reference to in vitro microcytotoxicity assays, no evidence of boosting of specific immune reactions was observed when patient lymphocytes, patient sera, or patient lymphocytes plus heat-decomplemented sera (ADCC) were tested against autologous glioma target cells, controlled with autologous brain target cells (Table 1).

The toxic effects usually anticipated with radiotherapy and chemotherapy, such as alopecia, transient nausea, and cyclic depression of the platelet and leukocyte peripheral blood counts, were tolerated well by patients in this study. However, 19% of our patients became symptomatic due to pulmonary interstitial fibrosis attributable to BCNU toxicity, and two patients in the VSG died from pulmonary insufficiency. We have extensively analyzed BCNU-induced pulmonary toxicity, and a discriminant function analysis has been developed that is capable of predicting this toxic side effect with greater than 80% accuracy.

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Test*</th>
<th>Months of Treatment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>glioblastoma</td>
<td>CC</td>
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<tr>
<td></td>
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<td></td>
<td>ADCC</td>
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<tr>
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<td>glioblastoma</td>
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<td>astrocytoma</td>
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<tr>
<td></td>
<td>ADCC</td>
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</tr>
</tbody>
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*CC = cellular cytotoxicity; HC = humoral cytotoxicity; and ADCC = antibody-dependent cellular cytotoxicity.
ASV Model Studies

Radiotherapy, Chemotherapy, and Levamisole. The results of our studies in the ASV model with therapies similar to the above clinical trial are summarized in Table 2. Fractionated radiotherapy (4600 rads total dose) and BCNU chemotherapy produced a significant increase in median survival (143%). There was no significant difference between the median survival time of animals treated with radiotherapy and BCNU chemotherapy alone and the median survival time of those treated with radiotherapy, chemotherapy, and levamisole (p > 0.05).

Specific and Nonspecific Active Immunotherapy Trials. The results of our studies involving other forms of immunotherapy in this model are shown in Table 3. Whereas single-agent immunotherapy was ineffective, multiple immunotherapy modalities did significantly improve survival times, with the most significant improvement (172%) occurring when all three forms of immunotherapy were combined with BCNU chemotherapy.

Discussion

Immunotherapeutic trials for patients with gliomas have been sparse. Bloom, et al., first attempted to induce an active immune response in a patient by subcutaneous implantation of living autologous glioblastoma cells. Although the tumor cells grew locally, there was no significant enhancement or shortening of the patient's survival time over what could have been predicted. Bloom also did not detect the appearance of complement-fixing serum antibodies directed against autologous glioma cells. A year later, Grace, et al., reported using this same approach in six patients with similar results. Although patient survival times were not significantly extended, there was a suggestion that an immunological reaction had occurred in some of the patients. For example, they observed the regression of local implants in four patients, two of whom subsequently developed positive DHR’s to extracts of their tumors. Trouillas and Laprus implanted glioma cells subcutaneously with or without BCG in a series of 20 patients. They also evaluated immunization with Freund’s complete adjuvant plus glioma cell extract distant to the implant site. Tumor growth was inhibited at the implant sites, and patients developed positive DHR’s to glioma extract. Unfortunately, this study included a small number of patients with insufficient immunological assays, and was uncontrolled so far as survival times were concerned. After intradermal immunization of 14 patients with autologous glioma extracts incorporated in Freund’s adjuvant, Trouillas reported immunodiffusion precipitation reactions between the patients’ sera and autologous glioma extracts. Preimmunization sera were negative. The postimmunization sera did not cross-react with extracts of meningioma or normal adult brain, but did have positive reactions with fetal brain extracts. Febvre, et al., reported DHR’s to lyophilized glioma cell lines in patients after immunization to their own gliomas. No reactions occurred to brain tissue itself, and preimmunization skin tests were all negative. In a prospective, randomized study, Bloom, et al., injected irradiated, autologous glioma cells subcutaneously up to three times in 27 patients. However, they were unable to demonstrate any improvement in survival or development of positive DHR’s to autologous glioma extracts. In this study, immune stimulants or adjuvants were not used, and no attempt was made to select candidates for immunotherapy who still had demonstrable general immunological reactivity. No other active clinical immunotherapy investigations with glioma patients have been reported.

Adjuvants alone have been tried in uncontrolled studies with BCG, and Corynebacterium parvum immunotherapy combined with mononuclear cells has had limited trials in patients with brain tumors. Leukocyte infusions into the cerebrospinal fluid circulation or into the tumor bed itself have been accomplished, but no evidence of immune reaction or of therapeutic effectiveness has been reported. It is apparent that all forms of immunotherapy of patients with gliomas have not received extensive trial in the past and have lacked detailed serial immune testing. Only those studies by Bloom, et al., and those of our own have been controlled and randomized, and none has previously attempted to prescreen patients for selection for immunotherapy.

In the last few years, we and others have gathered sufficient immunological data to permit construction of an immune profile of most patients with malignant gliomas at the time of surgical diagnosis: impaired DHR’s to recall skin test antigens; low numbers of circulating lymphocytes, particularly T-cells; impaired ability of lymphocytes to respond to mitogens (blastogenesis); elevated serum IgM levels; and poor antibody production in response to KLH (keyhole limpet hemocyanin) immunization. On the other hand, the capability of neutrophils from patients with gliomas to respond chemotaxically has been reported as normal. Furthermore, a direct correlation has been observed between the magnitude of perivascular inflammatory cells in histological sections of
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Anaplastic gliomas and survival times. At least half of these perivascular inflammatory cells have been classified as T-cells, and a considerable number of macrophages have been identified within malignant glioma tissues.

That something relevant may happen with the immune system in patients with gliomas is suggested by several other observations. In vitro blastogenesis of autologous lymphocytes has been demonstrated following co-incubation with glioma cells. Autologous lymphocytes have been seen to aggregate about glioma cells, and a small number of patients possess peripheral blood lymphocytes or serum antibodies capable of specific cytotoxicity directed against autologous glioma tissue. The presence of blocking factor in the serum of glioma patients has also been suggested. In summary, patients with gliomas appear to have 1) suboptimal cellular immune competence, 2) difficulty with initiation of new antibody responses, 3) the possible occurrence of blocking factor in the serum, and 4) minimal instances of specific anti-glioma-directed cellular or antibody responses (and of low magnitude when they do occur). Yet, there appear to be a number of inflammatory cells present in glioma tissues, and their presence may foretell a better prognosis. The question that remains unanswered is the relevance of these findings to the potential of an effective immune reaction by the patient to the glioma.

Levamisole, an agent used in the past for parasitic infections, has been recognized in the last 6 years as an immune stimulant, reported to restore suboptimal immune reactions, particularly those of a cellular nature in patients receiving this drug. In our clinical studies, the addition of levamisole therapy to the most effective conventional treatment for patients with anaplastic gliomas (radiotherapy with BCNU chemotherapy) failed to improve the survival times of these patients. Furthermore, the immune profile of patients with glioblastoma multiforme which has been described in the past did not appear to be altered by the administration of levamisole. There was no significant improvement in the DHR's, peripheral blood lymphocyte counts, or peripheral blood T-lymphocyte percentage, all of which tended to decline with time. The characteristic decline in serum IgM levels postoperatively was also not altered by the administration of levamisole. The only other clinical study at all comparable to this one was reported by Takakura, et al. In contrast to our findings, they did report an improvement in immune responses and survival times of patients with malignant gliomas who were treated with levamisole. However, this study was poorly controlled and included patients undergoing a plethora of other therapies, thus rendering interpretation of the actual effect of levamisole subject to serious criticism. Based on statistical multiparameter evaluation of our randomized prospective clinical trial, we conclude quite confidently that the addition of levamisole immunostimulation to conventional therapy for patients with malignant gliomas not only does not improve overall survival time, it also does not significantly boost general immune competence.

Further evidence of the lack of efficacy of levamisole immunostimulation was obtained from concomitant studies in an animal model system. The ASV-induced glioma model has the advantages of being autochthonous and histologically resembling the human anaplastic astrocytoma. Tumor induction by ASV in neonatal rats is rapid (2 weeks), but the animals survive 2 to 5 months after tumor induction if untreated. This time sequence permits administration of radiotherapy, chemotherapy, and immunotherapy before deaths begin to occur in the control group. We have used the ASV-induced glioma model in the past to study radiotherapy, BCNU chemotherapy, and combination protocols of treatment. These reports have shown meager improvement in survival time with BCNU chemotherapy alone, significant improvement in survival time with radiotherapy (with a dose-response relationship), and further improvement in survival with radiotherapy plus BCNU chemotherapy producing a synergistic effect. These results with this model system have closely paralleled those of the clinical trials undertaken by members of the Brain Tumor Study Group. The results in this model with radiotherapy, chemotherapy, and levamisole immunostimulation reported here also parallel the results of our clinical trial with levamisole.

One of the few encouraging reports of immunotherapy in humans with malignant gliomas has been that of Trouillas and Laprus, who immunized patients with glioblastoma multiforme with subcutaneous live autologous tumor cells combined with Freund's complete adjuvant. A potential hazard of such immunization is the growth of the subcutaneous glioma implants, with subsequent metastasis.

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### TABLE 3

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Percent ILS†</th>
<th>p-Value‡</th>
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</thead>
<tbody>
<tr>
<td>BCG</td>
<td>81</td>
<td>0.75§</td>
</tr>
<tr>
<td>sarcoma cells (live)</td>
<td>92</td>
<td>0.58§</td>
</tr>
<tr>
<td>levamisole</td>
<td>92</td>
<td>&gt; 0.10</td>
</tr>
<tr>
<td>BCG + sarcoma cells (live)</td>
<td>117</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>BCG + sarcoma cells (killed)</td>
<td>115</td>
<td>&lt; 0.05</td>
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<tr>
<td>BCG + sarcoma cells (live) + levamisole</td>
<td>137</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>BCG + sarcoma cells (live) + levamisole + BCNU</td>
<td>172</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*BCG = bacillus Calmette-Guerin.
†Experimental group median survival time/control median survival time × 100 = percent ILS (% increased length of survival).
‡Statistical comparison with control group.
§Data from our previous report.
nonviable cells could be substituted for living cells in active immunotherapy, this serious complication would be avoided. An important question is whether or not nonviable tumor cells would be as effective as living cells, since the antigenicity of the killed cells may be altered and their ability to elicit an anti-tumor response thereby decreased. On the other hand, cryptic antigenic sites on the tumor cells may be exposed during the process of rendering the cells nonviable; indeed, radiation has been shown to increase immunogenicity of mouse lymphoma cells and to cause the release from sarcoma cells of an antigenic component that was capable of activating a cellular immune response against the tumor. In our specific and non-specific immunotherapy studies with the ASV glioma model, we compared the efficacy of viable and radiation-killed allogeneic tumor cells in the immunotherapy of ASV-induced brain tumors and found no difference. For the first time, combined forms of immunotherapy alone were found to effectively prolong survival of ASV glioma-bearing rats. When BCNU chemotherapy was added to this combination, treatment showed further improvement in the anticipated survival time of these patients. This pilot clinical study will incorporate those combined modalities of therapy that have proven to be effective in the ASV glioma model which, to date, has exhibited a response pattern similar to that observed in the human.

Summary

Our results thus far in a clinical trial of the use of levamisole combined with radiotherapy and chemotherapy for the treatment of patients with malignant gliomas strongly suggest that levamisole does not provide further improvement in the anticipated survival time of these patients. In addition, there appears to be no significant alteration in the general or specific immune competence of patients undergoing levamisole treatment compared to that of patients treated with radiotherapy and chemotherapy alone. Our studies with the ASV glioma model have also demonstrated the effectiveness of radiotherapy and chemotherapy in prolonging survival time but, likewise, show no further improvement with the addition of levamisole immunostimulation. Further combined immunotherapy treatments in this model have resulted in significant improvement in survival time, which is further increased by combining immunotherapy with BCNU chemotherapy. Based on the results of these studies, we believe that a new pilot clinical study is warranted in order to investigate fully and more precisely combined immunotherapy as an adjunct to radiotherapy and BCNU chemotherapy in preselected patients with malignant gliomas.

References

8. Brooks WH, Roszman TL, Rogers AS: Impairment of
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