Role of Tumor Necrosis Factor in Flavone Acetic Acid-induced Tumor Vasculature Shutdown

Viswanath Mahadevan,1 Saleem T. A. Malik, Anthony Meager, Walter Fiers, Graham P. Lewis, and Ian R. Hart

ABSTRACT

Flavone acetic acid (FAA), a novel investigational antitumor agent, has been shown to cause early vascular shutdown in several experimental murine tumors, and this phenomenon is believed to be crucial to FAA's antitumor effects. However, the basis of this FAA-induced tumor vascular shutdown is unknown. In this study a radioactive tracer-clearance technique has been used as an objective indication of tumor blood flow to show that i.p. administered FAA induces a progressive and sustained reduction in blood flow in a colon 26 tumor growing s.c. in syngeneic mice. As early as 1 h after administration, there was a significant increase in the t½ clearance value for intratumorally injected 133Xe, reaching a peak at 3 h (117.3 ± 36.4 versus 7.8 ± 0.85 min for controls). Significant inhibition of blood flow was still apparent 48 h after a single injection of drug.

This FAA-induced vascular shutdown was virtually abolished in tumorbearing mice pretreated with an antiseraum against tumor necrosis factor, while no such effect was observed in controls pretreated with nonimmune serum (t½ of 10.8 ± 1.2 versus 65.6 ± 8.0 min for controls). Furthermore, in vitro FAA was seen to induce tumor necrosis factor secretion from murine peritoneal cells and splenocytes. These studies suggest that FAA-induced tumor vascular shutdown in the colon 26 tumor is mediated by tumor necrosis factor.

INTRODUCTION

FAA2 is a synthetic flavonoid whose in vivo antitumor activity was discovered in a National Cancer Institute screening program (1). FAA manifests potent activity against a variety of transplantable murine tumors growing s.c. as solid lesions but has little effect upon tumor cells in vitro or against most leukemia models (1–6). For a given tumor, FAA appears to be less effective against early stage disease than its more advanced better established counterpart (6, 7) and depends on the anatomical location of the tumor (8). The importance of anatomical location was demonstrated by Bibby et al. (8), who injected tumors into three sites (s.c., into the lungs, or i.p.) and monitored their response to i.p. drug. Tumors in the lungs or peritoneum were unresponsive to FAA, whereas the same tumors in the s.c. site were highly sensitive (8). These unusual features of FAA, coupled with a toxicity profile unlike that of most antineoplastic agents (9), have suggested that the drug may have an indirect mode of action.

One possible way FAA might exert its tumoricidal effects is by interrupting tumor blood supply. Thus, in a recent paper Bibby et al. (10) used a semiquantitative dye perfusion technique to show a marked reduction of tumor blood flow. Similarly, Evelhoch et al. (11), using nuclear magnetic resonance spec-

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1 To whom requests for reprints should be addressed, at Room 536, Imperial Cancer Research Fund Laboratories, P.O. Box 123, Lincoln's Inn Fields, London, WC2 3PX, England.
2 The abbreviations used are: FAA, flavone acetic acid; TNF, tumor necrosis factor; rMuTNF-α, recombinant murine tumor necrosis factor α, i.e., intratumoral; LPS, lipopolysaccharide; FCS, fetal calf serum; IFN-α/β, α/β interferon; PBS, phosphate-buffered saline.

troscopy, demonstrated a significant reduction in blood flow to an osteogenic sarcoma growing s.c. in mice. Most recently, Zwi et al. (12) have shown that blood flow failure is a major determinant in the antitumor activity of FAA. However, the mechanism underlying FAA-induced tumor blood flow reduction is a matter of speculation. On the basis of histological similarities between tumors treated with FAA and those treated with TNF (3), it has been proposed that FAA acts by inducing host effector cells to secrete vasoactive cytokines which may, in turn, mediate the observed changes in tumor vascularity.

In the present study we have adapted a radioactive xenon clearance technique, previously described by us (13, 14), to study the temporal sequence of blood flow changes in a s.c. colon 26 tumor model treated with i.p. FAA. The technique is based upon the Kety principle (15), which derives from the observation that inoculated radioactive tracer substance is lost exponentially from the injection site. The logarithm of the residual activity at the injection site plotted against time following injection yields a straight line, the slope of which represents tracer clearance and is a function of the rate of local blood flow. Clearance is expressed in terms of the half-time (t½), or the time taken for the initial count to fall by half. Thus the rate of blood flow bears an inverse relation to the t½ value.

Using this procedure, we now show that as early as 1 h after administration of FAA there is a marked and significant reduction in tumor blood flow, associated with hemorrhagic necrosis, which is apparent even up to 10 days later. This FAA-induced vascular shutdown was abrogated almost totally by pretreatment of mice with antiserum against tumor necrosis factor, suggesting that this cytokine plays a major role in mediating the effect of FAA against the colon 26 tumor.

MATERIALS AND METHODS

Animals. Young adult male BALB/c mice (weighing 24–28 g) were used in all experiments and were obtained from the Imperial Cancer Research Fund animal breeding unit (Clare Hall Laboratories, South Mimms, Herts, UK). All animal procedures were carried out under a Project Licence approved by the Home Office (London). Animals were housed individually in plastic cages in an air-conditioned room. Food and water were available ad libitum and a 12-h light/dark schedule was maintained.

Chemicals and Reagents. Radioactive xenon (133Xe) in sterile physiological saline (specific activity, 370 MBq in 3 ml) was purchased from Amersham International (Aylesbury, UK).

Clinically formulated FAA (LIPHA, Lyons, France), supplied as a lyophilized powder, was a generous gift from Dr. J. A. Double (Clinical Oncology Unit, University of Bradford, UK).

Recombinant murine tumor necrosis factor α had a specific activity of 7.5 × 10^5 units/mg (16).

Sheep anti-murine TNF-α antiserum was produced by immunizing a sheep with highly purified rMuTNF-α (Escherichia coli-derived; Gentech Inc., San Francisco, CA) in complete Freund's adjuvant. Antibody production was boosted at 6-week intervals by injection of rMuTNF-α until a highly neutralizing antiserum was obtained. A 10^5.5 dilution of this sheep anti-murine TNF-α antiserum reduced 10 cytotoxicity units of rMuTNF-α/ml to 1 cytotoxicity unit/ml. While a
neutralizing effect of the sheep antiserum was also demonstrated against rat TNF-α, it had no effect against other biologically active murine cytokines such as interleukin 1, interleukin 6, interferons, and tumor necrosis factor β.

Lipopolysaccharide (E. coli serotype 026:B6) was purchased from Sigma Chemical Company, Ltd. (Poole, UK).

Technique of Sponge Implantation. Circular, polyether, polyurethane sponge discs of 2-1.25 cm diameter x 0.6-0.3 cm thickness (Vitafoam Ltd., Manchester, UK) were sterilized in an autoclave at 125°C for 30 min prior to use. Mice were anesthetized, as were for all procedures excluding i.p. injections of FAA and physiological saline, by the i.m. injection of Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone; Janssen Pharmaceuticals) and Hypnovel (5 mg/ml midazolam hydrochloride; Roche), each at a dose of 0.5 ml/kg of body weight.

Using aseptic technique, a 1-cm dorsal, vertical, midline skin incision was made immediately proximal to the base of the tail. A dorsal s.c. pouch was fashioned 4-5 cm cephalad to the skin incision by gentle blunt dissection with a pair of curved artery forceps. A sterile sponge disc was then introduced through the skin incision into the s.c. pouch and positioned flat therein. The skin incision was closed with two interrupted 5-0 silk sutures and the animal was allowed to recover.

Tumor Establishment. Colon 26 cells (17) were maintained as confluent monolayers in tissue culture flasks containing Dulbecco’s modification of Eagle’s essential medium supplemented with 10% FCS and 1% L-glutamine. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cells were detached from tissue culture flasks using 0.1% trypsin/0.5 mM EDTA. The cell suspension was washed twice in PBS and resuspended to the appropriate concentration in PBS.

Preliminary experiments established that an inoculum of 1 x 10^5 to 1 x 10^6 cells in 100 μl PBS injected into the sponge 3 to 4 days after sponge implantation gave a 100% tumor take, with tumors being readily palpable between 12 and 14 days following tumor cell injection.

Blood Flow Measurement. Blood flow measurements were performed as described previously (13, 14). Briefly, the laboratory was equilibrated at 31°C and the animal was anesthetized. Ten μl of 133Xe solution in physiological saline were injected into the tumor-bearing sponge and the animal was positioned so that the sponge was 1 cm directly below a γ-scintillation probe. The washout of radioactivity was monitored at intervals of 1 min for 10 min on a programmable SR7 scaler rate meter (Nuclear Enterprises, Ltd., Reading, UK) connected in series to the γ-probe, and counts were printed automatically on an ESP 401 α-numeric and graphic printer (English Numbering Machines Ltd., London, UK). The counts were analyzed by a Macintosh-Apple computer program to derive the t½, for xenon clearance from the exponential curve fit.

FAA Treatment. Fifty-six animals received sponge implants followed 4 days later by injection, into the center of the sponge, of 1 x 10^9 colon 26 cells in 100 μl of PBS. Fourteen days after tumor cell injection (when all animals had palpable tumor around the sponge) 35 animals were killed for histological examination. Of the remaining 19 animals, 3 died (all 3 deaths occurred between 24 and 27 h after FAA treatment and were, therefore, designated as treatment-associated deaths), giving a treatment-associated mortality of 15.8%.

TNF Treatment. Sponges were implanted in 65 animals. Four days after sponge implantation, each animal received an injection, into the center of the sponge, of 1 x 10^5 colon 26 cells in 100 μl of PBS. Fourteen days after tumor cell injection (when all animals had palpable tumors), 60 animals were allocated arbitrarily to four groups (15 mice/group), to receive one of the following treatments: (a) i.v. rMuTNF-α (1.125 x 10^6 units in 150 μl of diluent/animal), (b) i.v. diluent (0.25% bovine serum albumin in PBS; 150 μl/animal), (c) i.t. rMuTNF-α (1.125 x 10^6 units in 150 μl of diluent/animal), or (d) i.t. diluent (150 μl/animal).

Within each treatment group, animals were further subdivided into three groups of 5 each and measurements of blood flow were made at 2, 4, and 24 h after treatment. The remaining 5 animals received no treatment and were used for baseline tumor blood flow measurements.

Pretreatment with Antiserum to TNF. Twenty-six animals received sponge implants followed 4 days later by an injection, into the center of the sponge, of 1 x 10^6 colon 26 cells in 100 μl of PBS. Fourteen days after tumor cell injection, when tumors were evident in all animals, 21 of the 26 tumor-bearing animals were allocated arbitrarily to three groups (7 mice/group). Two h prior to i.p. FAA administration (200 mg/kg), the three groups of animals were pretreated i.v. with one of the following reagents: (a) sheep anti-murine TNF-α antiserum (200 μl/animal), (b) nonimmune sheep serum (200 μl/animal), or (c) sterile physiological saline (200 μl/animal).

Blood flow was measured in the three groups 4 h following FAA administration and compared to that in the remaining 5 animals (no pretreatment, no FAA).

In Vitro Assay for TNF Activity. Splenocytes or peritoneal exudate cells were harvested from BALB/c mice using standard techniques (18, 19). Aliquots of 5 x 10^6 splenocytes (derived from a single mouse) or 5 x 10^6 peritoneal exudate cells (cells recovered from 3 mice were pooled) were plated in 500 μl RPMI 1640 medium containing 10% FCS. An additional 500 μl of this medium alone or medium containing 200 ng/ml lipopolysaccharide or 1 mg/ml FAA were added to these cell preparations, and cultures were incubated for varying times at 37°C. At indicated times, aliquots of supernatant were removed from these cultures and assayed for TNF activity against the TNF-sensitive WEHI 164 clone 13 cell line (18, 19).

The indicator WEHI 164 cell line was plated at 5 x 10^5 cells, in 100 μl/well RPMI/10% FCS, in 96-well dishes and allowed to adhere for 2 h at 37°C before 100 μl test supernatant, removed from the splenocyte or peritoneal exudate cell cultures, were added. Cultures were maintained for an additional 18 h at 37°C, washed 2 times with PBS to remove nonviable nonadherent cells, fixed in methanol for 5 min, dried, and stained with crystal violet (0.1% w/v). Plates were washed repeatedly with tap water until washings were colorless and stored dry until read. Stain was eluted by the addition of 200 μl dimethyl sulfoxide and absorbance at 620 nm was measured on a Titertek Multiscan (Flow Laboratories, Rickmansworth, UK). Triplet samples were read for each treatment and cytotoxicity was calculated as follows:

% cytotoxicity = \frac{A_{420} (medium only) - A_{420} (test sample)}{A_{420} (medium only)} \times 100

The specificity of the assay was confirmed by preincubating samples with the anti-murine TNF-α antiserum, a treatment which eliminated all activity. Standard curves generated using rMuTNF-α showed the
assay to be capable of recognizing levels of 1 pg/ml TNF (data not shown).

RESULTS

Tumor Blood Flow Measurements. Representative tumor blood flow, as estimated by the clearance of intratumorally injected $^{133}$Xe, is depicted in Fig. 1. Flow is expressed in terms of $t_v$ (min) calculated from the curve-fit equation. The effect of FAA (200 mg/kg) on tumor blood flow is shown in Table 1. Mean $t_v$ values from individual groups estimated at 1, 2, 3, 4, and 6 h after i.p. FAA injection were compared with that of a single control group (4 h after i.p. saline), while at 24 and 48 h separate control groups were used for comparison. There was no statistical difference between the three control values. A significant reduction in tumor blood flow was evident as early as 1 h after FAA treatment, reaching a maximum at 6 h, and even 10 days after drug injection blood flow in the tumor had not returned to normal values (Table 1).

Tumor Volume Measurements. A significant difference between mean tumor volumes of treated versus control groups was evident at 7 days after treatment (Table 2). Comparisons at later time points could not be made because within the next 2 days, all control animals were dead as a result of tumor burden. However, all FAA-treated animals remained alive for an additional 2 weeks and had mean tumor volumes at 14 and 21 days after FAA treatment which were approximately 50% of the control values found at 7 days (Table 2).

![Fig. 1. Representative xenon clearance curves from two animals, 4 h after i.p. FAA (●) and 4 h after i.p. saline (□). The $t_v$ values, as calculated from the computer-derived equation, are 152.9 and 10.4 min, respectively.](image)

**Table 1.** Effects of i.p. FAA (200 mg/kg) on clearance of i.t. injected $^{133}$Xe

<table>
<thead>
<tr>
<th>Hours following</th>
<th>$t_v$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p. FAA/saline</td>
<td>FAa</td>
</tr>
<tr>
<td>1</td>
<td>15.10 ± 2.10F (5)</td>
</tr>
<tr>
<td>2</td>
<td>117.30 ± 36.40F (5)</td>
</tr>
<tr>
<td>3</td>
<td>117.40 ± 19.65F (4)</td>
</tr>
<tr>
<td>4</td>
<td>7.8 ± 0.85 (5)</td>
</tr>
<tr>
<td>6</td>
<td>23.50 ± 36.40F (5)</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* Statistically significant (P < 0.001) difference in mean value compared with control values using Student’s t test.

Histology. The histological appearance of colon 26 tumor removed from an FAA-treated animal is represented in Fig. 2, which shows the alterations in blood vascularity associated with this drug.

TNF Treatment. The effects of TNF, given directly i.t. or i.v., on tumor blood flow are shown in Table 3. Both routes of administration resulted in significant and sustained reduction in tumor blood flow compared with the appropriate control group. The reduction in blood flow after i.v. TNF was considerably greater than that achieved with i.t. TNF. However, while the reduction induced by i.v. TNF lessened over the 24-h period under consideration (from 59.5 ± 6.8 to 24.6 ± 6.1 min), there was no such diminution of the effect of i.t. TNF (11.8 ± 0.5 min versus 14.0 ± 2.2 min).

Pretreatment with Anti-TNF Antiserum. By treating mice with antiserum to murine TNF, the reduction in blood flow induced by FAA could be abrogated almost completely. This experiment is illustrated in Table 4, where 4 h after FAA injection a $t_v$ value of 57.4 min in saline control or 65.6 min in nonimmune serum control animals was changed to 10.8 min after treatment with anti-TNF-α antiserum (Table 4). The effect remained apparent at 24 h, in that FAA was seen to have decreased tumor blood flow in the control, but not the treated, groups. Pretreatment with antiserum to murine TNF also abrogated totally the effect of FAA on tumor size. Thus, in an experiment totally independent from that shown in Table 2, the size of tumors by day 8 was 6.95 ± 0.7 cm³ in untreated controls. Animals pretreated with saline or nonimmune serum had tumor volumes of 1.4 ± 0.2 cm³ or 1.65 ± 0.2 cm³, respectively, indicating the tumor-shrinking effect of FAA. In contrast, animals that received FAA but had been pretreated with anti-TNF-α antiserum had tumor volumes of 6.4 ± 0.75 cm³, a value which was not significantly different from the no-FAA controls (Student’s t test; 6 or 7 animals/group).

In Vitro Assay for TNF. The results of a representative experiment, utilizing splenocytes, are given in Table 5. FAA appeared to be able to induce higher levels of TNF more rapidly than LPS (supernatant recovered after 30-min incubation had 38.6% cytotoxicity versus 4.7%; P < 0.01 Student’s t test) though after 4-h coincubation the levels of subsequent cytotoxicity evoked were very comparable (medium from LPS-conditioned splenocytes produced 40.3% cytotoxicity versus 33% cytotoxicity for FAA-conditioned splenocyte medium). The direct addition of 100 μl LPS (100 ng/ml) or FAA (500 μg/ml) to the indicator cells resulted in no detectable cytotoxicity. Similar results, both qualitatively and quantitatively, were obtained when peritoneal exudate cells were used instead of splenocytes (data not shown).

**DISCUSSION**

The technique used in the present study allows direct repeatable quantitative assessments of tumor blood flow to be made...
at various time points. This dynamic assessment shows that in s.c. colon 26 tumors of mice treated with i.p. FAA there is a clear and progressive reduction in blood flow, commencing as early as 1 h after FAA treatment (the earliest time point in our studies) and reaching a maximum between 3 and 4 h after treatment. The reduction in blood flow is maintained up to at least 10 days following treatment (the last time point in our studies).

Four h after drug administration, histological examination revealed an evident hemorrhagic necrosis. Apparent vascular engorgement and an increase in the diameter of tumor vessels was already detectable 2 h after the i.p. injection of FAA. These changes correlated with substantial benefits in terms of reduction in tumor volume (3.0 ± 0.18 cm³ versus 10.0 ± 0.5 cm³ for controls at 7 days after injection) and survival of tumor-bearing mice (Fig. 2 and Table 2). The first demonstration of an

<table>
<thead>
<tr>
<th>Hours following</th>
<th>i.v. TNF</th>
<th>i.v. diluent</th>
<th>i.t. TNF</th>
<th>i.t. diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59.5 ± 6.8*</td>
<td>6.3 ± 0.9 (5)</td>
<td>11.8 ± 0.5*</td>
<td>6.6 ± 0.7 (5)</td>
</tr>
<tr>
<td>4</td>
<td>48.4 ± 14.5*</td>
<td>6.9 ± 1.3 (5)</td>
<td>12.3 ± 2.8*</td>
<td>5.2 ± 0.6 (5)</td>
</tr>
<tr>
<td>24</td>
<td>24.6 ± 6.1*</td>
<td>6.4 ± 1.1 (5)</td>
<td>14.0 ± 2.2*</td>
<td>6.5 ± 0.8 (5)</td>
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</table>

*Mean ± SE.

Statistically significant differences in mean values compared with control values using Student’s t test (*P < 0.001, †P < 0.05, ‡P < 0.02).
association between FAA administration and a reduction in tumor blood flow was made by Evelhoch et al. (11). These authors, using nuclear magnetic resonance spectroscopy at the single time point of 4 h after drug injection, found a significant decrease in blood flow in a s.c. located carcinoma. The simpler indirect semiquantitative method of blood flow estimation by dye perfusion used by Bibby et al. (10) also revealed a sustained reduction in tumor blood flow; this reduction was shown to occur 2 h following FAA treatment (10). A positive correlation has been shown to exist between tumor vascular composition and responsiveness to FAA's tumoricidal effects in a s.c. murine colon carcinoma (7).

Recently Zwi et al. (12), using fluorescent dye perfusion coupled with a point-counting technique after tumor sectioning, were able to show that blood flow to an i.m. located EMT-6 murine tumor was reduced within 15 min of i.v. FAA administration. Moreover, 24 h after a single administration of drug, tumor but not normal vasculature was still almost completely closed down (12). Similarly Hill et al. (20), using **RbcI extraction as a measure of blood flow, demonstrated varying degrees of FAA-induced vascular flow inhibition in six experimental murine tumors. Further, there was a positive correlation between degree of blood flow inhibition and tumor response in terms of tumor growth retardation (20).

Thus the effects of FAA on tumor blood flow have been well documented and our results add to and extend these observations using a reproducible and quantitative technique. What has not been addressed in these studies is the mechanism underlying the FAA-induced vascular inhibition. Hydralazine, a potent hypotensive agent, does not mimic the antitumor efficacy of FAA, suggesting a simple hypotensive effect alone is insufficient to account for observed changes (10).

It has been proposed that FAA stimulates the release of cytokines from host inflammatory cells (6). The similarity of the histological changes evoked by FAA to those evoked by TNF-α (3) led us to investigate whether this specific cytokine was involved in the changes induced in our tumor model. We show in the present study that, whether given i.v. or i.t., recombinant TNF-α is capable of causing vascular shutdown (Table 3), while the pretreatment of mice with antisera to TNF-α abrogates completely the inhibition of tumor blood flow induced by subsequent administration of FAA (Table 4). Taken together with the finding that FAA induces splenocytes and peritoneal exudate cells to release material with TNF-α-like activity (Table 5), these results appear to indicate that TNF-α plays a major role in FAA-mediated vascular shutdown in the colon 26 tumor.

TNF is believed to exert its antitumor effects chiefly by inducing coagulation within the tumor vascular bed. Microvascular endothelium is an important target for TNF, and TNF has been shown to activate procoagulant functions on the endothelial cell surface (21). In their paper, Nawroth et al. (21) demonstrated that infusion of low concentrations of TNF into mice bearing Meth A sarcomas resulted in early fibrin deposition and formation of occlusive thrombi within the tumor vascular bed. This was associated with marked reduction in tumor perfusion (21). Interestingly, these changes were limited to the tumor vascular bed and did not occur in the adjacent normal microvasculature (21). Recently Murray et al. (22) showed that FAA induced a coagulopathy in both tumor-bearing and non-tumor-bearing mice and that the degree of the clotting abnormalities was dependent on the dose of FAA used. However, the intensity and duration of the coagulopathy was greater in tumor-bearing animals.

Mediators other than TNF may also be involved in FAA’s effects. Dvorak and Gresser (23) administered IFN-α/β locally to a s.c. tumor which was resistant to the direct antiproliferative activity of IFN-α/β, and this produced rapid damage of tumor, but not normal, microvasculature. Since FAA has been shown to induce IFN-α/β in experimental animals as well as in cancer patients undergoing clinical trials (24) it is possible that this cytokine too plays a part in effects on tumor vasculature. However, the virtually complete negation of FAA’s effects on blood flow by an antibody to TNF in our model suggests that, for this system at least, TNF-α is the major means whereby the flavonoid exerts its effects on tumor vasculature. This finding certainly is compatible with recent results reported by Mace et al. (25). These authors showed that the in vivo administration of FAA to BALB/c mice resulted in both an up-regulation of mRNA for TNF-α in splenic leukocytes and an increase in serum levels of TNF-α (25). Thus the antitumor effect of FAA against murine tumors may be partially or wholly mediated by the induction of TNF-α.

### Table 4 Effects of i.v. anti-TNF serum administered 2 h prior to i.p. FAA (200 mg/kg) on clearance of 11C-Xe

<table>
<thead>
<tr>
<th>Hours following i.p. FAA</th>
<th>Saline serum pretreatment</th>
<th>Nonimmune sheep serum pretreatment</th>
<th>Anti-TNF serum pretreatment</th>
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<tr>
<td>4</td>
<td>57.4 ± 7.0* (7)</td>
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<td>24</td>
<td>40.5 ± 6.2 (6)</td>
<td>31.6 ± 6.2 (6)</td>
<td>9.5 ± 1.2* (7)</td>
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</table>

* Mean ± SE.

Statistically significant differences in mean values compared with control values (nonimmune sheep serum-pretreated animals) using Student’s t test (* P < 0.001, ** P < 0.01).

### Table 5 Cytotoxicity of splenocyte-conditioned media against WEHI-164 cells

<table>
<thead>
<tr>
<th>Splenocyte incubation period</th>
<th>Cytotoxicity (%)</th>
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<tr>
<td>Medium alone</td>
<td>LPS (100 ng/ml)</td>
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<tr>
<td>30 min</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>4 h</td>
<td>40.3 ± 5</td>
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REFERENCES


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