Brief Definitive Report

Constitutive Expression of Interferon γ-inducible Protein 10 in Lymphoid Organs and Inducible Expression in T Cells and Thymocytes

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Summary

Interferon γ-inducible protein 10 (IP-10), a member of a family of small proinflammatory chemotactic polypeptides, is expressed in interferon γ-stimulated keratinocytes, macrophages, fibroblasts, and endothelial cells. Here we report that IP-10 is also expressed by activated but not resting T hybridoma cells, normal T cells, and thymocytes. Although resting lymphocytes did not synthesize IP-10, surprisingly high levels of IP-10 transcripts were found in lymphoid organs (spleen, thymus, and lymph nodes). Thymic and splenic stromal cells were found to express constitutively high levels of both IP-10 mRNA and protein, accounting for the high level of spontaneous expression in lymphoid tissue. Therefore, in addition to its role as a proinflammatory cytokine, IP-10 may participate in T cell effector function and perhaps T cell development.

Inducible protein 10 (IP-10) is an early-response gene expressed by activated human mononuclear cells, keratinocytes, fibroblasts, and endothelial cells (1, 2). IP-10 belongs to a family of genes that encode small peptides involved in proinflammatory and chemotactic processes during the immune response (reviewed in 3, 4). IP-10 was initially described as an early, transiently expressed, IFN-γ-inducible gene in a histiocytic lymphoma cell line with monocyte characteristics (1). IP-10 transcripts can also be induced in BALB/c 3T3 fibroblasts (5) and murine peritoneal macrophages (6, 7). Both human IP-10 and the murine homologue (C7 or CRG-2) (8, 9) encode a mature protein with a predicted M_r of ~10,000 that, like other members of this gene family, is secreted (2). The secreted product migrates with an M_r of 6,000–7,000 on SDS-PAGE (2, 10), and it has been suggested that posttranslational processing at the NH_2 terminus may account for the difference between the predicted and the actual size (11, 12).

The functional role of IP-10 has yet to be firmly established. Because of its inducibility by IFN-γ and its homology to platelet factor 4 and β-thromboglobulin, it is generally believed that IP-10 is involved in inflammatory responses, perhaps as a mediator of IFN-γ effects. Since IP-10 has recently been shown to be a chemoattractant for monocytes and T lymphocytes (13), IP-10 may function to increase local mononuclear cell infiltration during inflammatory processes such as delayed-type hypersensitivity and contact sensitivity. In addition, tumor cells transfected with an IP-10 expression construct have been shown to be rejected more efficiently than nonsecreting tumors (10), perhaps by potentiating a T cell response. IP-10 has also been shown to inhibit colony formation of bone marrow progenitor cells (11), suggesting its effects may be quite pleiotropic.

During the course of experiments designed to identify T cell genes induced by cellular activation, we isolated IP-10 cDNA from an anti-TCR stimulated T cell hybridoma cDNA library. This prompted us to examine normal lymphoid tissues for IP-10 expression.

Materials and Methods

Cells and Reagents. 2B4.11 is a cytochrome *c*-specific T cell hybridoma (14) maintained in RPMI/10% FCS (BioFluids Inc., Rockville, MD). TEC and TEPI are thymic epithelial cell lines kindly provided by Dr. Ada Kruisbeek (Netherlands Cancer Institute, Amsterdam, The Netherlands) (15). 145-2C11(2C11) is a hamster anti-mouse CD3-ε mAb (16), and G7 is a mitogenic rat anti-mouse Thy-1 mAb (17). $ZnSO_4$, cycloheximide (CHX), PMA, ionomycin, and Con A, were obtained from Sigma Chemical Co. (St. Louis, MO). Cyclosporin A (CsA) was purchased from Sandoz Pharmaceuticals Corp. (East Hanover, NJ) and stored as a stock solution in 50% ethanol.

Preparation of Plasmid DNA. A cDNA clone of mouse IP-10, CRG-2, was kindly provided by Dr. Joshua Farber (Johns Hopkins

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University, Baltimore, MD). Plasmid DNA was prepared according to Maniatis (18). A HindIII/XbaI fragment of this plasmid containing ~851 bp was used as a probe in Northern blot analysis. ^{32}P-labeled probes were prepared by random primer labeling using the T7 Quick Prime labeling kit from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), according to the manufacturer's instructions.

Preparation of Stromal Cells. Stromal cells were prepared by crushing thymi or spleens with a syringe plunger and washing the remaining particulate matter several times to eliminate as many lymphocytes as possible. For protein secretion experiments, the stromal elements were further treated with collagenase (0.6 mg/ml in PBS with 1% FCS) for 1 h at 37°C. The single cell preparations obtained after such treatments were still 70%–80% Thy-1$^+$.

Preparation of RNA and Northern Analysis. In experiments with 2B4.11 cells, 50 × 10^6 cells were stimulated in Falcon Integrid 150 × 25 mm tissue culture dishes (model no. 3025; Falcon Labware, Becton Dickinson & Co., Oxnard, CA) coated or not with purified 2C11 (10 μg/ml in PBS) (19) and in the presence or absence of CHX, CsA, or $ZnSO_4$. After the indicated times, cells were harvested and total RNA extracted using the RNAzol (Tel-Test, Inc., Friendswood, TX) method as per manufacturer's instructions. Both splenocytes and thymocytes were stimulated in dishes coated with 2C11 or medium containing the indicated stimulus, harvested after 3 h at 37°C, and RNA was extracted as above. In the case of normal tissue, isolated material was homogenized (Brinkmann Instruments Co., Westbury, NY) and RNA extracted using the RNAzol procedure. Equal amounts of RNA (30 μg) were denatured, subjected to electrophoresis on a 1% agarose-formaldehyde gel, and blotted by capillary transfer to Hybond-N membranes (Amersham Corp., Arlington Heights, Illinois) (18). The membranes were prehybridized at 42°C in a solution containing 50% formamide, 12.5× Denhardt's solution, 5× SSC, 0.1% SDS, 0.05 mg/ml denatured salmon sperm DNA, and 200 μg/ml yeast RNA. After 4–8 h of prehybridization, a labeled denatured probe was added and hybridization was carried out for another 16–20 h. The blots were generally washed twice with 2× SSC/0.1% SDS for 10 min at room temperature and three times for 20 min at 55°C, although similar results were obtained when blots were washed at higher stringency (1× SSC/0.1% SDS at 65°C). The blots were exposed using X-OMAT AR X-ray film (Eastman Kodak Co., Rochester, NY) with DuPont Co. (Wilmington, DE) Lightning-Plus intensifying screens at −70°C. Expression of GAPDH (kindly provided by Dr. F. Mushinski, National Cancer Institute [NCI]) was always assessed on the same blots as a control for the quantity of mRNA loaded in each lane. For the experiments involving CsA, after probing for IP-10, the blots were stripped and probed again with IL-2 cDNA (a gift of Dr. F. Mushinski, NCI).

Secretion of IP-10. To analyze the secretion of IP-10, cells were cultured in serum-free medium (HL-1; Ventrex Laboratories, Portland, ME) for 20–24 h at a concentration of 5 × 10^5 cells/ml (2B4.11) or 2–2.25 × 10^6/ml (thymocytes and thymic stromal cells). Supernatants were concentrated ~10-fold on a Centricon-3 (Amicon, Danvers, MA) and supernatants electrophoresed on a 15% Tris-Tricine gel (20). After transfer onto Immobilon-P (Millipore Corp., Bedford, MA), the blots were probed with an anti-IP-10 rabbit antiserum (10), developed with alkaline phosphatase-conjugated donkey anti-rabbit antiserum (Jackson ImmunoResearch Laboratories, West Grove, PA), and detected using the chemiluminescent substrate, Lumiphos-530 (Boehringer Mannheim, Indianapolis, IN), as described (21).

Results

Induction and Regulation of IP-10 Expression in 2B4.11 Cells. A subtractive hybridization analysis undertaken to identify genes induced during T cell activation led to the isolation of a cDNA clone that was identified as the previously characterized murine homolog of the IFN-γ–inducible cytokine, IP-10 (data not shown). Northern blot analysis revealed that IP-10 mRNA is expressed at very low levels in resting murine 2B4.11 T hybridoma cells (Fig. 1). Upon stimulation via the TCR, the level of IP-10 mRNA increased within 1 h, continued to rise until 3–4 h after stimulation, and then rapidly declined. These kinetics are similar to that observed in activated macrophages (6). IP-10 has been shown to be a macrophage early response gene (i.e., its expression can be transcriptionally upregulated in the absence of continued protein synthesis). To determine if this is true in T cells, 2B4.11 cells were stimulated in the presence of the protein synthesis inhibitor CHX. CHX was added to resting or stimulated 2B4.11 cells and IP-10 expression assessed 3 h later (Fig. 2 *A*). The inducible expression of IP-10 was not prevented by CHX: instead, the level of mRNA was enhanced in both nonstimulated and 2C11-activated cells (compare lanes *1* and *2* with *5* and *6*). The 3′ untranslated region of IP-10 contains the AUUUA motif thought to bind labile proteins that promote message destabilization (22). Inhibiting synthesis of these labile proteins often leads to superinduction of short-lived messages containing this motif. Since CHX increases IP-10 transcript levels in both unstimulated and stimulated 2B4.11 cells, these data are consistent with the possibility that steady state transcript levels are at least partially regulated by message destabilization.

The T cell hybridoma 2B4.11 undergoes programmed cell death when activated (14, 19). One of the hallmarks of T cell apoptosis is the activation of DNA endonucleases (23), and perhaps RNA endonucleases (24). Since endonucleases may be involved in the destabilization of mRNA containing AUUUA motifs (22), we stimulated 2B4.11 cells with 2C11 in the presence of inhibitors of apoptosis to see if IP-10 transcript levels would be superinduced. CsA, a potent inhibitor of IL-2 and IFN-γ transcription (25) as well as activation-induced apoptosis, did not inhibit the 2C11-induced increase in IP-10 expression (Fig. 2 *A*), although in the same experiment IL-2 mRNA induction was completely inhibited (Fig.

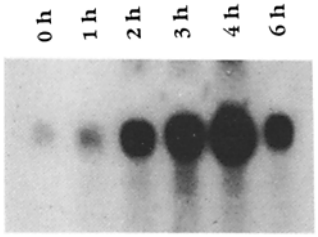

Figure 1. Kinetics of IP-10 induction in 2B4.11. 2B4.11 cells (50 × 10^6) were stimulated on 2C11-coated (10 μg/ml) 150-mm Petri dish plates for 0–6 h. At the indicated times, cells were harvested and total RNA extracted. 30 μg of each RNA sample was subjected to Northern blot analysis using an XbaI/HindIII fragment of IP-10 as a probe. GAPDH was used as a control for RNA loading (data not shown).

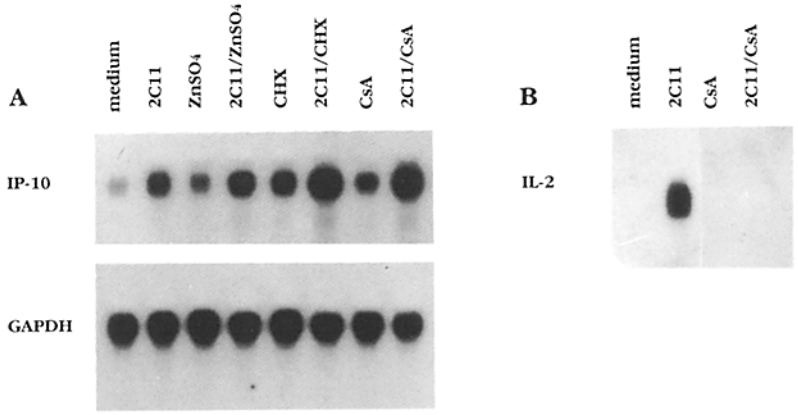

Figure 2. Effect of different treatments on the induction of IP-10 by 2B4.11 cells. (*A*) 2B4.11 cells were stimulated for 3 h on plates that were previously treated or not with 2C11 (10 μg/ml), 66 μM $ZnSO_4$, 500 ng/ml CHX (lanes *e* and *f*), 50 ng/ml CsA, or combinations of these. 25 μg of total RNA was separated in a 1% agarose gel, transferred to nylon membranes and probed for IP-10 or GAPDH message. (*B*) The blot shown in *A* was stripped and the lanes representing 2B4.11 cells cultured in medium with or without 2C11 and with or without CsA were reprobed for IL-2 mRNA.

2 *B*). CsA actually enhanced IP-10 expression in unstimulated and 2C11-stimulated 2B4.11 cells, suggesting that CsA may function to inhibit the destabilization of IP-10 mRNA, either directly via an endonuclease or indirectly by inhibiting other proteins involved in the destabilization process. In addition, we stimulated 2B4.11 cells in the presence of Zn^{2+}, an inhibitor of Ca^{2+}/Mg^{2+}-dependent endonucleases that prevents DNA fragmentation in thymocyte nuclei ([23] and Yang, Y., and J. D. Ashwell, unpublished observation) and 2B4.11 cells (Zacharchuk, C., and J. D. Ashwell, unpublished observation). When added to resting 2B4.11 cells, Zn^{2+} caused a small increase in the level of IP-10 mRNA, and no enhancement of activation-induced IP-10 expression (Fig. 2). Therefore CsA, but not Zn^{2+}, augments resting and TCR-mediated increases in IP-10 mRNA levels, perhaps by influencing message stability.

Expression of IP-10 in Normal Tissues. A survey of IP-10 transcript expression in normal mouse tissues was undertaken. A small amount of IP-10 mRNA was detected in kidney and lung, and none in heart or brain (Fig. 3), confirming observations that constitutive expression of IP-10 in normal tissue is generally quite low (26). Unexpectedly, Northern blot analysis revealed that IP-10 is constitutively expressed at high levels in lymphoid organs (thymus, lymph node, and spleen) as well as in liver. To determine if activated lymphoid cells were responsible for high levels of IP-10 mRNA, RNA from isolated thymocytes was analyzed for IP-10 expression. As shown in Fig. 4, no IP-10 was detected in resting thymocytes. Stimulation with Con A did not increase IP-10 mRNA, and PMA alone induced only a small amount. Concomitant stimulation with PMA and the Ca^{2+} ionophore ionomycin, or activation with a mitogenic anti-Thy-1 mAb, resulted in large increases in IP-10 transcripts. Stimulation with an anti-CD3 antibody, 2C11, also induced IP-10 mRNA at 3 h, but in repeated experiments there was too much RNA degradation (presumably due to induction of apoptosis) to accurately quantitate the levels (data not shown).

Since expression in resting thymocytes seemed unlikely to account for the high level of IP-10 transcripts in the thymus, thymic stroma was analyzed. As shown in Fig. 5, the level of expression in thymic stroma was as high as that in whole thymus (Fig. 5 *A*). Two thymic epithelial cell lines, TEC and TEPI, both expressed high levels of IP-10 mRNA (Fig. 5 *B*), confirming that thymic epithelial cells are capable of expressing IP-10. Similar results were obtained with spleen preparations (Fig. 6); nonactivated splenocytes did not express IL-10, but expression was induced by activation with 2C11. RNA made from splenic stroma did hybridize with

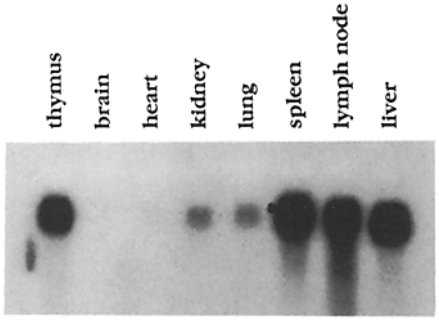

Figure 3. Expression of IP-10 in tissues. Thymus, brain, heart, kidney, lung, spleen, lymph node, and liver were homogenized and total RNA extracted. 30 μg of each RNA was separated on a 1% denaturing agarose gel by electrophoresis, transferred to nylon membranes, and hybridized with an XbaI/HindIII IP-10 fragment.

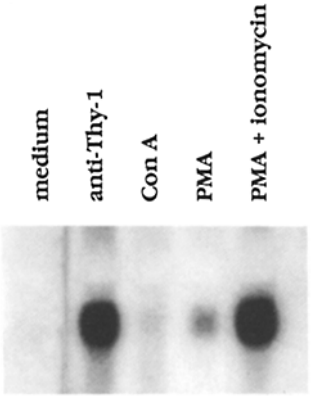

Figure 4. Expression of IP-10 in activated, but not resting, thymocytes. Thymocytes (10^8/dish) were cultured for 3 h with medium alone, 10 μg/ml G7, 5 μg/ml Con A, 10 ng/ml PMA, or 1 ng PMA plus 0.5 μg/ml ionomycin. After stimulation, total RNA was made and Northern blot analysis performed. 30 μg of each RNA sample were loaded in each lane.

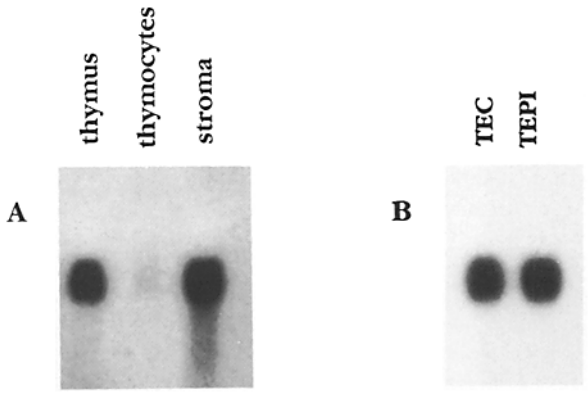

Figure 5. Expression of IP-10 by thymus and stromal cell lines. Whole thymus, thymocytes, and thymic stroma were isolated, total RNA extracted by the RNAzol procedure, and subjected to Northern blot analysis using the IP-10 probe (*A*). TEC and TEPI stromal cell lines were grown in RPMI/10% FCS. RNA prepared from each cell line (30 μg/lane) was used for Northern analysis (*B*).

the IP-10 probe, although once again degradation of RNA from this source made quantitation difficult. Together, the data indicate that IP-10 is constitutively expressed by thymic and splenic stromal cells, and that expression can be induced by activation in thymocytes and peripheral T cells.

Secretion of IP-10 by 2B4.11 Cells and Thymic Stroma. The ability of different cell populations to secrete IP-10 protein was also assessed. Using the antimurine IP-10 antiserum for immunoblotting, IP-10 appears as a doublet (10). Consistent with their respective IP-10 transcript levels, 2B4.11 cells secreted much more IP-10 when activated by anti-CD3. Likewise, whereas thymic stromal cells produced easily detectable IP-10 protein, thymocytes produced very little immunoreactive material (Fig. 7). Thus, both activated T cells and thymic stromal cells, in the absence of overt stimulation, synthesize and secrete the IP-10 chemokine.

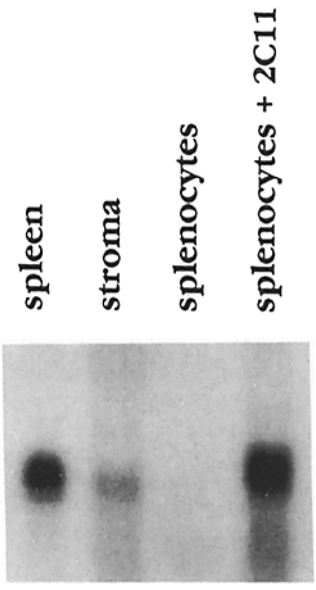

Figure 6. Expression of IP-10 transcripts by splenocytes and splenic stroma. Whole spleen, splenocytes, and splenic stroma were isolated, total RNA extracted by the RNAzol procedure, and subjected to Northern blot analysis using the IP-10 cDNA probe.

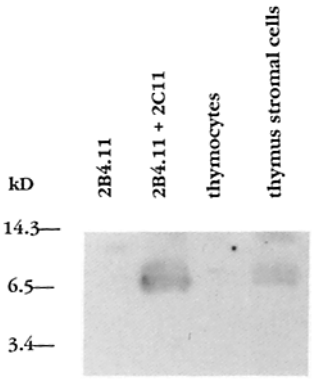

Figure 7. Thymic stroma and activated 2B4.11 cells secrete IP-10. 2B4.11 cells were cultured in serum-free medium alone or with plate-bound 2C11; thymocytes and thymic stromal cells were cultured in serum-free medium alone. After 20 h, the supernatants were concentrated and aliquots removed for assay. Secreted products from 2.5×10^5 2B4.11 cells or 1.25×10^6 thymocytes and thymic stromal cells were assayed for the presence of IP-10.

Discussion

IP-10 is a member of a family of proinflammatory and chemotactic cytokines expressed by activated macrophages, fibroblasts, keratinocytes, and endothelial cells (1, 5–8). The results in this paper demonstrate that IP-10 is also expressed by activated T cells. Although IP-10 was first described as an IFN-γ–induced monokine, it is unlikely that IFN-γ is involved in 2C11-induced expression of IP-10 in T cells. First, IP-10 expression is rapid (within 1–2 h). Second, if expression of IP-10 transcripts depended upon the synthesis of IFN-γ, it would be expected that inhibition of protein synthesis would inhibit IP-10 expression; CHX actually led to increased accumulation of IP-10 transcripts. Third, no IFN-γ activity was detected in the supernatants of 2C11-activated 2B4.11 cells (data not shown), indicating that activation of 2B4 neither induces IFN-γ synthesis nor causes the release of presynthesized cytokine. Therefore, it seems likely that activation-induced expression of IP-10 is a direct consequence of TCR-mediated activation rather than secondary to a secreted lymphokine.

Like macrophages, expression of IP-10 in T cells increases rapidly after stimulation, reaches a maximum in 3–4 h, then declines. This transient expression has been attributed to the presence of an AUUUA motif in the 3′ untranslated region of the transcript that leads to message destabilization (27). Rapid degradation of RNAs bearing such sequences is due to recognition of this specific region by RNA-binding proteins (28, 29) that either target the RNA for destabilization or lead directly to its destruction. CHX not only blocks degradation conferred by these AU-rich sequences, but can cause superinduction of gene expression (22). In agreement with these observations, treatment of 2B4.11 with CHX results in increased expression of IP-10 in both unstimulated and stimulated cells.

Although induction of IP-10 expression in 2B4.11 is increased by TCR-dependent activation, CsA, a potent inhibitor of many activation responses in T cells, actually increases IP-10 mRNA under the same conditions that completely inhibit the induction of IL-2 mRNA. The immunosuppressive effect of CsA on T cell proliferation is via a Ca^{2+}-sensitive pathway involving calcineurin, a calmodulin-dependent protein phosphatase (30, 31). Our results suggest that IP-10 expression in 2C11-stimulated cells is not dependent on this

pathway. Since CsA increases IP-10 expression in both unstimulated and stimulated cells, it is possible that the expression of a protein involved in message destabilization is inhibited by CsA. It should be noted that the opposite result (inhibition of IP-10 expression by CsA) was observed by another group using PHA-activated human T lymphocytes (32). The reason for the discrepancy between the data is not known, but could be due to differences in the source and purity of the cells (a murine T cell hybridoma vs. semipurified human peripheral blood T cells), the stimulus (anti-CD3 vs. PHA or PHA plus PMA), or the duration of stimulation (3 vs. 12 h).

Constitutive expression of IP-10 in tissues such as liver, spleen, lymph node, and thymus was unexpected. It is possible that the presence of IP-10 transcripts in liver is due to the presence of activated macrophages. The other nonlymphoid tissues examined, lung, heart, kidney and brain, express few if any IP-10 transcripts. Despite its inducibility upon activation, the constitutive expression of IP-10 observed in whole thymus does not seem to be due to the presence of activated thymocytes, as isolated thymocytes do not have detectable levels of IP-10 mRNA. The majority of the constitutively expressed IP-10 in thymus seems to be due to epithelial cells, as both isolated thymic stromal cells and thymic epithelial cell lines spontaneously express high levels of IP-10. These data suggest that the thymic stromal cells constitutively express IP-10 or are induced to do so under normal physiological conditions. Similar results were obtained when splenocytes were isolated from splenic stroma. Although the physiological role of IP-10 remains to be determined, it probably participates in inflammatory processes and may be a mediator of macrophage function (33, 34). Furthermore, IP-10 has recently been shown to be a chemoattractant for monocytes and activated T cells (13). It is possible that in addition to its participation in macrophage function, IP-10 could function in the thymus and participate in T cell–mediated immune responses.

We thank Dr. J. Oppenheim and Dr. D. Taub for a critical review of the manuscript, and Dr. J. Farber for providing IP-10 cDNA.

This work was supported in part by a postdoctoral fellowship from the Brazilian Research Council (C. R. Gattass), a National Research Service Award F32 CA-09162 (L. B. King), and the Damon Runyon-Walter Winchell Cancer Fund (A. D. Luster).

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Received for publication 23 November 1993 and in revised form 4 January 1994.

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