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Elevated levels of GM-CSF and IL-1 in the serum, peritoneal and pleural cavities of GM-CSF transgenic mice

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SUMMARY

Levels of granulocyte–macrophage colony-stimulating factor (GM-CSF) in the peritoneal and pleural cavity fluid of two lines of GM-CSF transgenic mice were abnormally high (up to 120,000 U/ml), often exceeded the elevated serum GM-CSF levels in these mice and correlated with the increased number of macrophages present. In the peritoneal fluid, the only significant elevations of IL-1 levels were seen in moribund male-line transgenic mice. In contrast, IL-1 levels in pleural cavity fluid of male-line transgenic mice were clearly higher than those in littermate control mice or female-line transgenic mice. In male-line transgenic mice, IL-1 levels in both peritoneal and pleural cavities correlated with local macrophage numbers. Endotoxin was detectable in the peritoneal cavity fluid from some mice of all types but did not correlate with elevated IL-1 levels. No correlation was observed between levels of GM-CSF or IL-1 in the peritoneal cavity and the development of fibrotic nodules in the peritoneal cavity or gut congestion, two lesions common in male-line GM-CSF transgenic mice. The data suggest that the elevated levels of IL-1 in GM-CSF transgenic mice may be the consequence of stimulation by GM-CSF of IL-1 production by the elevated numbers of macrophages in these mice.

INTRODUCTION

The granulocyte–macrophage colony-stimulating factor (GM-CSF) is a haemopoietic regulatory glycoprotein with the ability to stimulate the formation of granulocytes, macrophages and eosinophils (Metcalf *et al.*, 1986). However, CSF can also stimulate various functional activities of the mature cells, including the production by monocyte–macrophages of interleukin-1 (IL-1) (Morrissey *et al.*, 1987, 1988). Injection of GM-CSF intraperitoneally into mice greatly elevates the number of peritoneal macrophages, granulocytes and eosinophils, with evidence of functional activation of the macrophages (Metcalf *et al.*, 1987).

Two lines of GM-CSF transgenic mice have been generated by insertion of two copies of the GM-CSF gene with a Moloney virus long-terminal repeat promoter (Lang *et al.*, 1987). In one line (female-derived) insertion of the transgenes is on the X chromosome and in the other line (male-derived), insertion is on an unidentified autosomal chromosome. Mice of both lines exhibit elevated serum levels of GM-CSF, elevated numbers of peritoneal and pleural macrophages and die prematurely, often with inflammatory foci containing macrophages in skeletal muscle and other locations.

IL-1 has been implicated as an initiator of the production of mediators involved in inflammatory responses (Mizel, 1982; Dinarello, 1984; Beck *et al.*, 1986; Mochizuki *et al.*, 1987; Kasahara *et al.*, 1988; Zsebo *et al*, 1988). Production of IL-1 by macrophages was shown to be stimulated by GM-CSF (Morrissey *et al.*, 1987, 1988). An analysis has therefore been performed of IL-1 levels in the peritoneal and pleural cavities and serum of transgenic mice, comparing these with GM-CSF levels and cell numbers in the cavities of these mice.

MATERIALS AND METHODS

GM-CSF transgenic mice

The production and maintenance of the two GM-CSF transgenic lines has been described elsewhere (Lang *et al.*, 1987). The lines were developed and held under specific pathogen-free conditions. All transgenic mice develop ocular opacity and can be classified unambiguously at weaning.

Serum collection

Mice were anaesthetized then bled from the axillary vessels. After clotting, the sera were removed, diluted 1:4 in saline then millipore filtered and stored for assay at 4°.

Abbreviations: CTLL, cytotoxic T lymphocyte cell line; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-1, interleukin 1.

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Peritoneal and pleural washouts

After each mouse was killed by exsanguination, the peritoneal and pleural cavity of each mouse was injected separately with a 2 ml volume of 5% serum saline using a No. 25 needle. The thorax and abdomen were gently massaged to ensure adequate mixing of the injected fluid with resident cells. Each cavity was then opened sequentially and the fluid harvested gently using a blunt plastic Pasteur pipette. The harvested volume was noted to determine whether peritoneal ascites fluid or a pleural effusion was present. Cell counts were performed and cytocentrifuge preparations made of each cell suspension. The supernatant fluid was removed after centrifugation at 3000 *g* for 5 min, millipore-filtered, then stored for assay at 4°.

GM-CSF assays

Levels of GM-CSF in serum, peritoneal or pleural washout fluids were assayed using 5-μl volumes of serial two-fold dilutions in microwell cultures of 200 cells of the GM-CSF-responsive line FDC-P1. The specificity of this assay in GM-CSF transgenic mice and its calibration have been documented previously (Metcalf, 1988). Mixing experiments with known amounts of GM-CSF failed to reveal the presence of inhibitors in negative sera or washout fluids that would have prevented the detection of GM-CSF.

IL-1 assays

IL-1 activity was measured using a two-stage bioassay incorporating the murine thymoma line EL4 6·1 NOB-1 and CTLL cells (Gearing *et al.*, 1987). Briefly samples were incubated with 1×10^5 NOB-1 cells in RPMI-1640 containing 5% fetal calf serum (FCS) (total volume 200 μl/well). After 18 hr culture supernatants were harvested, frozen and thawed to kill any NOB-1 cells carried over, and 40 μl of serial dilutions of each incubated with 4×10^3 CTLL cells in RPMI-1640 containing 5% FCS (total volume 100 μl/well). After 18 hr each sample was pulsed with 0·5 μCi of tritiated thymidine. Cells were harvested 4 hr later and incorporated radioactivity determined by liquid scintillation counting. Although the assay is unresponsive to endotoxin, there is a possibility that T-cell growth factors in samples could stimulate proliferation of the CTLL cells. To exclude this possibility 50 samples which gave positive responses in the assay were also assayed on CTLL cells alone. None of these samples stimulated the CTLL cells. The NOB-1 assay can respond to murine tumour necrosis factor (TNF)α (A. Gearing, unpublished data). However, maximal responses are never more than 50% of those seen with IL-1 and a minimum of 1 ng/ml is required to give a detectable response. The NOB-1 cells do not respond to GM-CSF or IL-6, another macrophage product.

Results were calculated in units after comparison with the National Institute for Biological Standards and Control interim reference reagent for IL-1α. The dilutions involved and the sensitivity of the assay of approximately 100–200 fg/ml (0·01–0·02 U/ml) resulted in a minimum detectable level of IL-1 of 0·1 U/ml for serum, 0·6 U/ml for peritoneal fluid and 0·8 U/ml for pleural fluid.

Endotoxin assays

Assays of the level of endotoxin in peritoneal and pleural washout fluids were performed using the Limulus assay (Levin & Bang, 1968) (limulus amebocyte assay, Commonwealth Serum Laboratories, Melbourne; detection limit 0·1 ng/ml). Mixing experiments excluded the possible presence of inhibitors able to prevent the detection of endotoxin.

Observations

After collection of serum and the peritoneal and pleural cavity fluids, the mice were autopsied and histological examination performed of all major organs.

RESULTS

An analysis was made of 22 littermate control mice, 54 healthy or moribund male-line transgenic mice and 17 moribund female-line transgenic mice aged between 2 and 5 months. Control littermate mice in this age range were killed, then injected after death either intraperitoneally or intrathoracically with a known volume of ^{125}I-labelled ovalbumin. Counts on fluid reharvested from these cavities established that the approximate volume of fluid normally present in the peritoneal cavity was 70 μl and in the pleural cavity was 50 μl. These figures were used to calculate the concentration of GM-CSF and IL-1 in resident fluid from the levels observed in washouts made using an injected volume of 2 ml. In seven peritoneal cavities and two pleural cavities from male-line transgenic mice an ascites or pleural effusion was observed and this was taken into account in the calculations of GM-CSF and IL-1 concentrations.

Comparison of GM-CSF levels in serum, peritoneal and pleural cavities

With three exceptions, littermate mice had no detectable GM-CSF in either the serum or peritoneal cavity. The GM-CSF transgenic mice of both lines differed sharply in having high, if variable, serum GM-CSF levels. Overall, there was a correlation between levels of GM-CSF in the serum and the peritoneal cavity. There were 20 examples where peritoneal GM-CSF levels were below those in serum and 30 examples where peritoneal cavity concentrations were higher than in the serum, sometimes markedly higher. These latter examples suggested that the peritoneal cavity cells in transgenic mice may be a major source of serum GM-CSF. This is supported by the observations that the most frequent cell type in transgenic peritoneal populations is an enlarged macrophage and that transgenic peritoneal cells actively transcribe the GM-CSF transgene (Lang *et al.*, 1987).

Peritoneal cell counts in the transgenic mice studied ranged from 2 to 684×10^6 cells and, as shown in Fig. 1, a correlation was observed between peritoneal GM-CSF concentrations and total cellularity, GM-CSF levels being significantly higher in cavities containing more than 10×10^6 cells than in cavities containing fewer than 10×10^6 cells ($\chi^2 = 10{\cdot}9$, $P < 0{\cdot}01$).

GM-CSF was detected in only two pleural cavity fluids from littermate control mice. In pleural fluids from transgenic mice, in 13 instances GM-CSF levels were lower than serum GM-CSF levels and in 21 instances were higher, sometimes markedly higher, than serum GM-CSF levels. Pleural cavity cell counts in the transgenic mice ranged from 1 to 113×10^6 cells and again a correlation was observed between total cells or total macrophages and observed GM-CSF levels, GM-CSF levels being significantly higher in cavities containing more than 10×10^6 cells than in cavities containing fewer than 10×10^6 cells (Fig. 1)

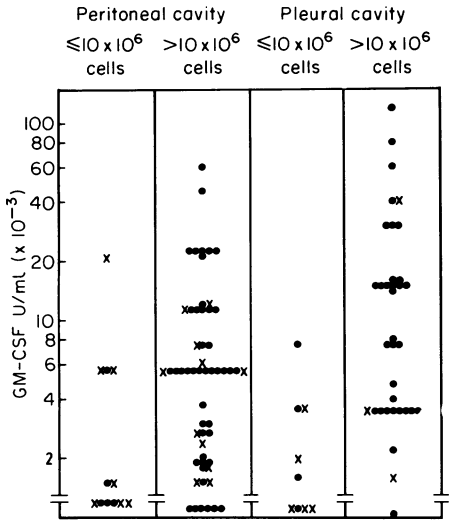

Figure 1. Correlation in individual mice between GM-CSF levels in the peritoneal or pleural cavity and total cellularity in the cavities. (●) Male-line transgenic mice; (X), female-line transgenic mice.

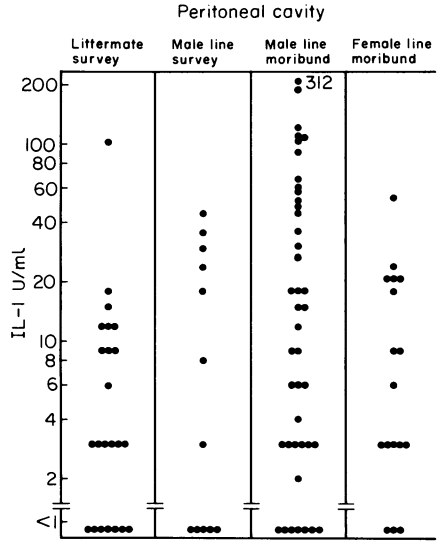

Figure 2. IL-1 levels in the peritoneal cavity of littermate mice versus male-line and female-line transgenic mice.

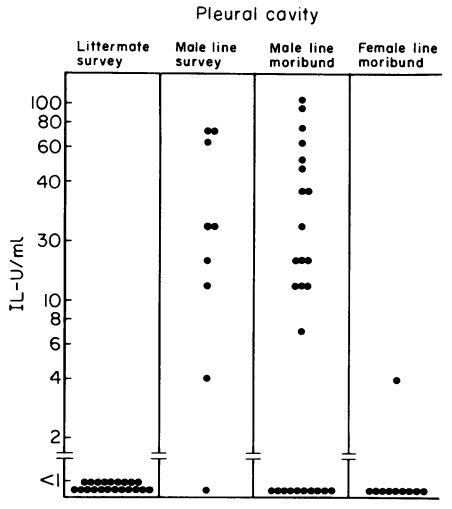

Figure 3. IL-1 levels in the pleural cavity of littermate versus male-line and female-line transgenic mice.

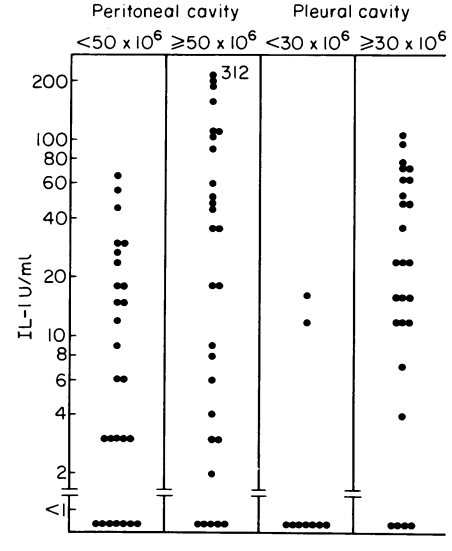

Figure 4. Correlation in individual male-line transgenic mice between IL-1 levels in the peritoneal or pleural cavity and total cellularity in the cavities.

($\chi^2 = 10{\cdot}2$, $P < 0{\cdot}01$). In individual mice there was a fair correspondence between peritoneal and pleural GM-CSF levels, 61% of paired assays being within a two-fold difference.

In the peritoneal and pleural cell populations of the transgenic mice, the dominant cells (more than 85%) were enlarged macrophages. This was the only cell population whose numbers correlated with observed GM-CSF concentrations.

IL-1 levels in peritoneal and pleural fluid

Peritoneal fluids from most littermate and transgenic mice contained detectable IL-1. As shown in Fig. 2, the only group in which IL-1 levels were significantly higher than in littermate control mice were moribund male-line transgenic mice ($\chi^2 = 6{\cdot}7$, $P < 0{\cdot}01$).

IL-1 levels in pleural fluids showed a quite different pattern. No IL-1 was detectable in either littermate pleural fluid or (with one exception) in fluid from moribund female-line mice. Levels of IL-1 both in healthy and moribund male-line transgenic pleural fluids were significantly elevated above those in littermate mice (Fig. 3) ($\chi^2 = 21{\cdot}0$, $P < 0{\cdot}01$; $\chi^2 = 16{\cdot}9$, $P < 0{\cdot}01$, respectively).

In both peritoneal and pleural cavity specimens from male-line mice, a significant correlation was observed between IL-1 levels and total cell counts (Fig. 4) ($\chi^2 = 6{\cdot}8$, $P < 0{\cdot}01$; $\chi^2 = 9{\cdot}3$,

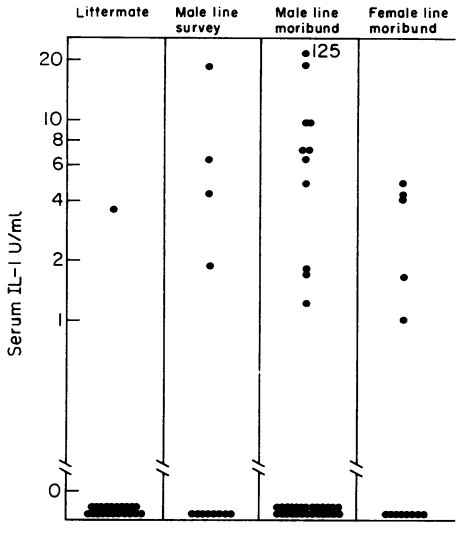

Figure 5. Serum IL-1 levels in littermate mice compared with those in male-line and female-line transgenic mice. Detection limit in the assay was 0·1 U/ml.

$P < 0{\cdot}01$, respectively). As in the case of GM-CSF levels, no correlation was noted between IL-1 levels and the numbers of neutrophils, lymphocytes, eosinophils or mast cells in the populations, the only correlation being between the numbers of the dominant macrophage population and IL-1 levels.

In individual transgenic mice, levels of IL-1 in the peritoneal cavity correlated significantly with GM-CSF levels in the cavity but no such correlation was observed in the pleural cavity between the levels of IL-1 and GM-CSF.

Serum IL-1 levels

Serum IL-1 levels were determined on the mice included in the above analysis of peritoneal and pleural cavities. As shown in Fig. 5, only one of 22 littermate sera (5%) contained detectable IL-1 but IL-1 was detectable, in some cases at very high levels, in 15 of 49 sera (31%) from male-line transgenic mice and five of 13 sera (38%) from female-line transgenic mice.

In individual mice, no correlations were observed between serum IL-1 levels and serum GM-CSF levels or with peritoneal and pleural cellularity or IL-1 levels.

Endotoxin levels in peritoneal or pleural cavity fluid

Assays for endotoxin content were performed on some of the fluids from peritoneal and pleural cavities. Endotoxin was not detectable in any of 16 pleural cavity fluids from transgenic mice but endotoxin (0·1–2·0 ng/ml) was detected in peritoneal fluids from seven of 12 littermate and seven of 20 transgenic mice. No correlation was observed in individual mice between the levels of endotoxin, GM-CSF or IL-1.

Correlation of IL-1 levels with lesions in the peritoneal or pleural cavity

Male-line transgenic mice develop a 56% incidence of nodules in the peritoneal cavity composed of macrophages and fibroblast-like cells and an 18% incidence of similar lesions in the pleural cavity (Metcalf & Moore, 1988). Female-line transgenic mice never develop such lesions. The other major disease state shown by GM-CSF transgenic mice involving these cavities is the development in moribund mice of a blackened small bowel and upper part of the colon associated with vascular congestion and microscopic bleeding into the gut lumen. This disease occurs in 34% of female-line transgenic mice but in only 10% of male-line mice (Metcalf & Moore, 1988). In the transgenic mice surveyed in this study, the presence of elevated IL-1 or endotoxin levels in the peritoneal or pleural cavities did not correlate either with the presence of abdominal or pleural nodules or with gut congestion.

DISCUSSION

While mice of the two transgenic lines exhibit approximately similar elevations of serum GM-CSF levels, their peritoneal and pleural populations differ significantly in that macrophage numbers are higher in male-line mice than in female-line mice and differ in morphology (Lang *et al.*, 1987; Metcalf & Moore, 1988).

The present studies have shown that levels of GM-CSF in the resident peritoneal and pleural fluid are as elevated as levels in the serum and, more often than not, somewhat higher. These observations indicate that macrophages resident in these cavities are exposed to stimulation by very high local GM-CSF concentrations.

Production of IL-1 *in vitro* by macrophages has been shown to be stimulated by GM-CSF (Morrissey *et al.*, 1987, 1988). GM-CSF transgenic macrophages express higher than normal numbers of GM-CSF membrane receptors (N. A. Nicola and D. Metcalf, unpublished data) and thus have the potential of being stimulated by the excessive local concentrations of GM-CSF. This appears to be a reasonable basis for the observed elevated levels of IL-1 in the pleural cavity of GM-CSF transgenic mice. That transgenic macrophages are the likely source of the elevated IL-1 levels noted in this study has been supported by *in situ* hybridization studies showing increased transcription of IL-1 mRNA by these cells (R. A. Cuthbertson and R. A. Lang, manuscript in preparation).

The situation in the peritoneal cavity is less clear since IL-1 was also present in the cavities of some normal mice. The assay system used for IL-1 does not respond directly to stimulation by endotoxin. However, endotoxin was detectable in some peritoneal cavity fluids and, since as little as 100 pg/ml endotoxin can stimulate IL-1 production by monocytes (Poole *et al.*, 1988), some of the IL-1 observed in peritoneal fluids may have been induced by endotoxin. However, examples were observed where high IL-1 levels were present but no endotoxin and, in such mice, stimulation by the high GM-CSF levels appears to be a reasonable explanation.

It was of interest that some female-line transgenic mice had high peritoneal IL-1 levels but no detectable IL-1 in the pleural cavity. Furthermore, IL-1 was not necessarily detectable in the serum even when high concentrations were present in the peritoneal cavity. This suggests that IL-1 may be a more locally confined regulator than GM-CSF and not necessarily be detectable in the serum even when high local IL-1 concentrations are present in the body.

The present studies show that the GM-CSF transgenic mouse can exhibit high local and/or circulating levels of IL-1.

The mice are therefore useful models for detecting some of the consequences of prolonged elevations of IL-1. While no correlations were observed in individual mice between IL-1 levels and local disease states in the abdominal or pleural cavity, local IL-1 has been implicated in the development of some types of inflammatory lesions (Beck *et al.*, 1986; Kasahara *et al.*, 1988; Dinarello, 1984) and could be responsible in part for the inflammatory lesions developing in GM-CSF transgenic mice.

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