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In vivo Interleukin-2 Activated Sheep Lung Lymph Lymphocytes Increase Ovine Vascular Endothelial Permeability by Non-lytic Mechanisms

Daniel E. Bechard, R. Paul Fairman, Daniel B. Hinshaw, Alpha A. Fowler and Frederick L. Glauser

Therapeutic doses of recombinant interleukin-2 (rIL-2) often result in systemic toxicity consistent with increased vascular permeability. rIL-2 activated lymphocytes (IALs) may produce endothelial dysfunction and have cytolytic potential. However, much of the data on IAL cytotoxicity comes from the use of *in vitro* activated IALs. Alternatively, rIL-2 may enhance permeability directly or via release of various cytokines by host effector cells. The cytotoxicity of *in vivo* activated lung lymph lymphocytes has been studied in an ovine model of rIL-2 toxicity. The *in vivo* IALs had no significant endothelial cytolysis at effector to target ratios of 100:1. However, the *in vivo* IALs increased endothelial monolayer permeability to albumin, dependent on the concentration of IALs. rIL-2 induced no endothelial cytolysis or permeability alterations at doses of 10^5 and 2×10^5 U/ml, respectively. These findings suggest that the acute endothelial dysfunction characteristic of the vascular leak syndrome is not due to rIL-2 directly, but is mediated by *in vivo* IALs via non-cytolytic mechanisms and/or the release of secondary cytokines in response to rIL-2.
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INTRODUCTION

ADOPTIVE IMMUNOTHERAPY, the systemic infusion of recombinant interleukin-2 (rIL-2) alone or in combination with *in vitro* expanded rIL-2 activated lymphocytes (IALs) (also called lymphokine activated killer [LAK] cells), is used to treat various animal and human tumours [1, 2]. Tumoricidal activity depends upon the dose and duration of treatment of rIL-2, as well as on the number of *in vitro* IALs infused [3]. Host toxicity (fever, weight gain, malaise, hypotension, azotaemia and respiratory distress) is common. In man, a "vascular leak syndrome" often develops which limits the dose and duration of therapy [4]. The development of increased systemic and pulmonary vascular permeability during rIL-2 adoptive immunotherapy has been substantiated in several animal models [5–8].

Whether the rIL-2, the *in vivo* or *in vitro* IALs, the release of secondary cytokines or the activation of other host cells is responsible for this vascular leak syndrome is unclear. Initially, high doses of rIL-2 were felt to be the cause, although the exact mechanism(s) remain unclear and the available data are contradictory. rIL-2 infusion into isolated, perfused lungs increases microvascular permeability in some [6] but not all models [9]. rIL-2 has also been reported to have no direct

deleterious effects on a variety of endothelial and epithelial cell lines, and does not increase albumin flux across cultured endothelial monolayers [10]. However, although controversial, *in vitro* produced IALs may increase permeability in isolated, perfused lung models and endothelial monolayer systems in various species [10]. The cytotoxic potential of *in vivo* IALs, which may be functionally different from *in vitro* activated lymphocytes, or exposure of endothelial cell monolayers to high doses of rIL-2, which may be present in the micro-environment of cell–cell interfaces, has not been investigated. We have examined the effect of *in vivo* produced lung lymphatic IALs and high doses of rIL-2 on endothelial monolayer permeability and endothelial cytolysis.

MATERIALS AND METHODS

Ovine endothelial cell preparation

Freshly harvested pulmonary arteries were rinsed in Hank's balanced salt solution (HBSS)(Gibco) with 1% penicillin/streptomycin (P/S). The arterial segment was opened, the luminal surface gently scraped with a scalpel blade and the endothelial layer dispersed into a single cell suspension and transferred to T-25 Primaria flasks (Falcon) containing growth medium consisting of Dulbecco's modified essential medium (DMEM)(Gibco) supplemented with 1% P/S, 5% fetal bovine serum (FBS)(Gibco) and endothelial cell growth supplement (25 μg/ml)(Sigma). At confluence, primary cultures were mechanically scraped and subcultured into sterile 25 cm^2 flasks (Corning). Before all assays, endothelial cells were verified by cobble-stone morphology under inverted phase microscopy [11] and by factor VIII immunofluorescent staining [12]. All assays were done on cell passages 5–12.

rIL-2

Vials of human rIL-2 and sterile excipient were provided by the Cetus Corporation (Emeryville). This rIL-2 is immunologically and biologically similar to native human IL-2 with the exception of two aminoacids and lack of glycosylation [13]. rIL-2 was reconstituted in sterile water to 3×10^6 U/ml and then adjusted to the desired concentration.

In vivo produced IALs

The protocol was approved by the university's animal use committee. Activated lung lymph lymphocytes were produced in the sheep chronic lung lymph fistula model [5, 14]. Baseline lung lymph was collected over 2 h into a sterile, iced heparinised tube. The cellular constituents were collected by centrifugation 400 *g* at 4°C for 10 min; the cell pellet was resuspended in growth media. Aliquots were removed for viability testing by trypan blue exclusion, and cell differential count after Dif-Quik staining (Sigma) of a cytocentrifuge sample. The cellular suspension was adjusted to the required density in growth medium for the particular assay (see below). The sheep was then given a constant infusion of rIL-2 (3×10^5 U/kg per day) over the next 72 h. Harvesting of the cellular components of the lung lymph drainage was repeated at the completion of the rIL-2 infusion and after a 48 h recovery/washout period. Each animal (with baseline, rIL-2 infused, and recovery lymphocytes) served as its own control.

All cellular assays were done in the presence of rIL-2 (30 U/ml) to insure lymphocyte activation was maintained [15].

Endothelial cytolysis

A ^{51}Cr release assay was used to assess the cytolytic potential of IALs and rIL-2 against cultured ovine endothelial cells. On the day before assay, endothelial cells were labelled by adding 11 MBq ^{51}Cr (New England Nuclear) to a culture flask of endothelial cells nearing confluence. The cells were incubated overnight at 37°C in 5% CO_2. On the day of assay, the endothelial monolayer was washed with fresh growth medium three times to remove non-incorporated label, incubated for 30 min, and re-washed three times. The labelled cells were gently trypsinised (0.05% trypsin, Gibco), washed and resuspended at 5×10^4/ml in culture medium without FBS. 100 μl endothelial suspension (5000 cells) was placed into 96-well round-bottom microtitre plates (Corning) and 100 μl lung lymph lymphocytes adjusted to yield the desired effector to target ratio were added. For rIL-2 cytolytic studies, varying concentrations of rIL-2 in growth medium were added. The assay was initiated by low-speed centrifugation of the plates, followed by incubation for 4 h at 37°C in 5% CO_2. The supernatants were harvested (Skatron-Titertek) and counted in a Compugamma counter (LKB). Appropriate controls for spontaneous release were performed by incubating labelled endothelial cells in medium or cell-free lung lymph. Equivalent concentrations of rIL-2 excipient (mannitol 60 mg per vial and sodium dodecyl sulphate, 54 mg per vial) were incubated with labelled endothelial cells to control for possible toxicity from these constituents. Total cellular label was measured by incubation in 0.1 mol/l HCl to lyse the cells (confirmed by inverted phase microscopy). All trials were run in triplicate. Specific lysis was determined by: (test well − spontaneous release) × 100 ÷ (total lysate − spontaneous release).

Endothelial monolayer permeability

Permeability was assessed by the passage of radiolabelled albumin across confluent endothelial monolayers [16]. Cultivated ovine pulmonary artery endothelial cells were gently trypsinised, pelleted by centrifugation and resuspended at 8×10^5/ml in growth medium. 0.5 ml cell suspension (4×10^5 cells) was seeded onto sterile, commercially prepared polycarbonate membranes (tissue-treated, 0.4 μm pore, 24 mm^2 Transwell system; Costar). This system consists of two compartments separated by a polycarbonate membrane on which endothelial monolayers were grown. Growth medium was added to the luminal and abluminal chambers (final volume 1.6 and 2.5 ml, respectively). The medium was changed at 24 and every 72 h thereafter. In preliminary experiments, monolayer confluence was determined by light microscopy after silver nitrate staining: 4×10^5 endothelial cells consistently became confluent by day 5 in culture. All assays were run on day 5.

On the day of the experiment the medium in the abluminal chamber was replaced by DMEM (1% P/S) without FBS (final volume 2.4 ml). Varying concentrations of lung lymph cells (with 30 U/ml rIL-2) or rIL-2 in growth medium were added to the luminal well of the assay system at time zero (final volume 1.6 ml). Tracer amounts of [^{125}I]albumin (New England Nuclear) were added to the luminal chamber. Growth medium

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Table 1. *Lymph-fluid characteristics*

Time	Lymph/plasma protein	Cells per μl	Lymphocytes (%)
Baseline	0.59 (0.28)	7.31×10^4 (0.91)	98.5 (2.1)
rIL-2 infusion	0.91 (0.25)*	6.01×10^4 (0.92)	97.6 (1.3)
Recovery	0.54 (0.21)	15.1×10^4 (2.1)*	96.6 (0.9)

Mean (S.D.). *$P < 0.01$ compared with baseline.

alone served as the negative control and was run with every experiment. Positive controls consisted of 0.05% trypsin in DMEM. Tracer flux experiments across the naked membrane (no endothelial monolayer) were also done. Experimental groups consisted of: (1) membrane alone, (2) monolayer with medium alone, (3) monolayer with trypsin, (4) monolayer with varying lung lymph cell densities and (5) monolayer with varying concentrations of rIL-2.

[^{125}I]albumin flux across confluent endothelial cell monolayers was used as an index of endothelial permeability. The appearance of [^{125}I]albumin in the abluminal chamber was measured, after thorough mixing, every 10 min for 60 min. Luminal compartment samples were obtained at the beginning of each experiment (time zero). [^{125}I]albumin clearance (C_{alb})(μ) was calculated as $C_{alb} = V_L \times L/U$ where V_L is the volume (μl) in the abluminal chamber at the time of sampling, L is the [^{125}I]albumin activity in the abluminal chamber (counts per min per μl), and U is the [^{125}I]albumin activity in the luminal chamber. Albumin clearance rate (μl/min) was calculated by least-squares linear regression [16]. To control for inter-experimental variability, all results were also expressed as relative change in permeability of the experimental monolayer vs. the monolayer control (monolayer plus media) for that group of wells (permeability index).

Statistics

Unpaired t tests were used. $P < 0.01$ was considered significant.

RESULTS

The characteristics of the ovine lung lymph are shown in Table 1. Following the 3 day rIL-2 infusion, recovered lung lymph mononuclear cells were "lymphoblastic" in appearance with a high nuclear-to-cytoplasmic ratio, open nuclear chromatin

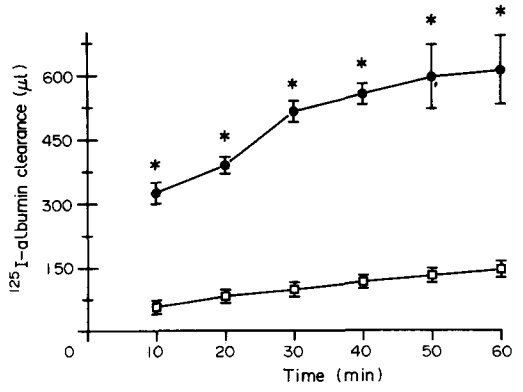

Fig. 1. Albumin flux across endothelial monolayers (□) and polycarbonate filter (●), $n = 10$. * $P < 0.001$.

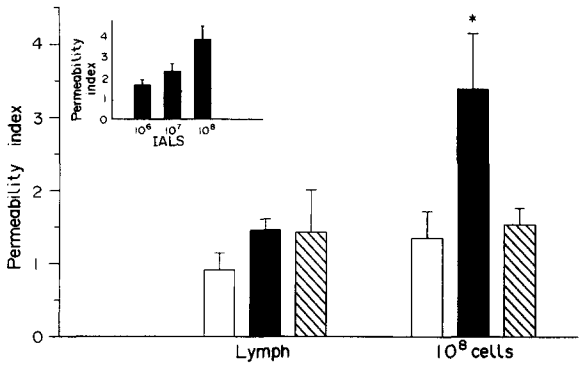

Fig. 2. Permeability index in cell-free lymph or lymph cells. □ = baseline, ■ = rIL-2 treated and ▨ = recovery, $n = 6$. Inset = IAL effect on permeability, $n = 6$. * $P < 0.001$.

and many nuclei containing single prominent nucleoli. In addition, these "activated" lymphocytes demonstrated a doubling in the mean cell surface area and were esterase-negative, consistent with rIL-2 activated lymphocytes demonstrated in lung histological sections [14, 17]. This activated morphology had reverted to normal by the recovery period.

The spontaneous release of ^{51}Cr from labelled endothelial cells was 3–4% per h. When exposed to lung lymph lymphocytes, the endothelial cells showed only minimal specific cytolytic activity (under 5%), even at effector to target ratios of 100:1. Cell-free lung lymph had no effect on endothelial viability at any time (results not shown). There was no significant endothelial cell cytolysis at concentrations of rIL-2 up to 10^5 U/ml ($n = 16$) or with equivalent concentrations of excipient (results not shown).

Endothelial monolayers, compared with the polycarbonate filter, restricted [^{125}I]albumin flux ($n = 10$) with mean clearance rates of 1.74 (S.E. 0.29) and 5.94 (1.42), respectively (Fig. 1). Endothelial permeability remained normal when exposed to baseline lymph cells at concentrations up to 10^8 cells per well. Compared with controls, IALs increased permeability 4-fold in a cell density dependent manner ($P < 0.001$) (Fig. 2, insert). Endothelial permeability was normal with the recovery lymph cells. Cell-free lymph had no effect on endothelial permeability at any time (Fig. 2). Lung lymph lymphocytes from animals treated with excipient had no effect on endothelial permeability (results not shown). No significant alteration in the clearance rates of [^{125}I]albumin was observed following exposure to concentrations of 10^5 U/ml (2.42 [0.38] μl/min) and 2×10^5 U/ml rIL-2 (2.40 [0.26] μl/min) compared with the untreated endothelial monolayer (1.74 [0.29] μl/min). Trypsin significantly increased [^{125}I]albumin flux (7.4 [0.27] μl/min) compared with control and rIL-2 treated monolayers (Fig. 3).

DISCUSSION

Loss of endothelial integrity by cytolysis is a common mechanism causing increased vascular permeability. However, increased endothelial permeability may also result from nonlytic mechanisms such as altering the cytoskeleton [16, 18]. In our present study, *in vivo* produced lung lymph IALs increased, in a density dependent fashion, endothelial monolayer permeability to albumin. Neither baseline nor recovery lung lymph lymphocytes altered albumin flux. Cell-free lymph did not alter endothelial permeability which suggests soluble mediators have no role or are below their effective concentration ranges in lung

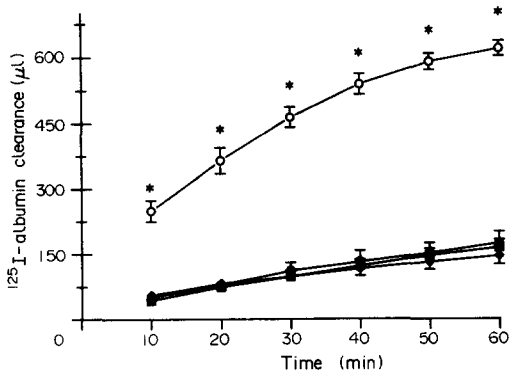

Fig. 3. Albumin clearance across endothelial monolayers. ◆ = medium, ● = 10^5 and ■ = 2×10^5 U/ml rIL-2 and ○ = 0.05% trypsin, $n = 10$. * $P < 0.001$.

lymph. Damle and Doyle [10] reported that *in vitro* human IALs increased human umbilical vein endothelial permeability. When corrected for differences in cell number per well and surface area of the monolayer, permeability changes they saw were approximately 9-fold greater than those reported here. The difference in permeability may be related to different assay conditions, or more likely, to different degrees of lymphocyte activation generated by *in vitro* compared with *in vivo* rIL-2 exposure. With similar doses of rIL-2, Gambacorti-Passerini *et al.* [19] found a 4–10-fold increase in *in vitro* exposed lymphocyte activation compared with *in vivo* exposure in melanoma patients undergoing rIL-2 immunotherapy.

Endothelial permeability with high levels of rIL-2 (2×10^5 U/ml) did not increase albumin clearance. Similar findings have been reported, although the maximum rIL-2 dose used was 10^4 U/ml [10, 20]. In addition, fluorescence microscopy of endothelial cells exposed to 10^5 U/ml rIL-2 for 1 h demonstrated no alterations in microfilament or microtubule organization (D.E.B. and D.B.H., unpublished). These observations further support our findings that rIL-2 does not acutely enhance vascular permeability. The inability of high rIL-2 doses to increase vascular permeability or alter cytoskeletal architecture confirms previous studies and ensures that transient peak plasma levels associated with bolus infusion or high concentration of IL-2 at the effector–target interface are not responsible for the observed increases in endothelial permeability.

We examined whether the increased permeability induced by *in vivo* IALs was the result of cytolysis. Animals receiving rIL-2 have esterase-negative, large lymphoid-like cells (histologically similar to *in vitro* IALs) within the microvasculature of target organs (i.e. lung, kidney, spleen) [3, 5, 10, 17, 21]. We [22] and others [23, 24] have reported direct *in vitro* IAL cytotoxicity towards cultured endothelial cells. Therefore, it has been assumed that *in vivo* or *in vitro* produced IALs alter vascular permeability via direct cytolytic mechanisms. The lack of cytolytic activity by the *in vivo* IALs in the present study was surprising. At an effector to target ratio of 100:1, there was no significant endothelial cytolysis even though the lung lymph IALs demonstrated morphological changes compatible with activation [14], similar to lymphocytes activated *in vitro* [25, 26] and observed in the perivascular regions and small lymphatics of pulmonary tissue in sheep after 3 day rIL-2 infusions [8, 17].

This lack of cytolytic activity could be due to several factors: (1) Loss of IAL activation following isolation from the lymph. This is unlikely since the IALs were continually exposed to rIL-2 (30 U/ml) and the morphological changes persisted after a 4 h incubation in assay medium containing this cytokine. (2) Loss of a critical circulating factor during the isolation steps. Cytolytic assays done with IALs resuspended in the lung lymph demonstrated no lytic activity, verifying that the absence of cytolysis was not due to the removal/loss of a necessary soluble factor (results not shown). (3) Cytolytic activation of lymphocytes requires rIL-2 exposure greater than 72 h. Tumour cell lysis by *in vitro* IALs is proportional to incubation time with significant increases in tumour target lysis not appearing until after at least 72 h of rIL-2 exposure [23, 27, 28]. Duke *et al.* [24] found that sheep peripheral blood lymphocytes required a 4 day incubation with 1000 U/ml rIL-2 to express cytolytic activity against the K562 tumour line. Lymphocyte cytolytic activity is also proportional to the dose of rIL-2 present during incubation [27]. Although IAL cytolytic activation at doses of 10–20 U/ml has been reported [27], rIL-2 concentrations of 1000 U/ml over 5 days are typically required [4, 29]. It is possible that *in vivo* rIL-2 levels in our model were not high enough to produce cytolytic activity in the IALs, although increased vascular permeability was clearly present. (4) There may be differences in degrees of activation between *in vivo* compared with *in vitro* rIL-2 exposure [19]. In addition, non-specific cytolytic activity may be altered by a variety of circulating factors *in vivo* (e.g. prostaglandin cyclic adenosine monophosphate) [30, 31]. (5) The source of lymphocytes may also determine the degree of activation by rIL-2. Pulmonary derived ovine lymphocytes may not develop the cytolytic potential seen in peripheral blood lymphocytes following incubation with rIL-2 [24, 32].

Despite this lack of cytolytic activity by the lung lymph IALs, rIL-2 induces the vascular leak syndrome in the lung lymph fistula model [8, 14, 17]. Damle *et al.* [9] reported IALs treated with dexamethasone had suppressed cytolytic activity, yet enhancement of endothelial permeability persisted. Thus the way IALs lyse tumour cells may be distinct from that responsible for increasing endothelial permeability.

rIL-2 may acutely enhance microsvascular permeability by acting directly upon vascular endothelium [6, 7]. Conflicting evidence in *in vivo* murine models indicates that a cellular immunocompetent state must be present during the rIL-2 infusion for the vascular leak syndrome to develop [33]. Others have reported no morphological changes of cultured endothelium or expression of endothelial cell surface activation antigens following exposure to rIL-2 [34, 35]. Direct rIL-2 induced cytolysis may not occur at peak rIL-2 plasma levels associated with bolus infusion or at the high concentrations of this cytokine in the effector–target interface. However, we could not completely exclude direct toxicity since we evaluated the acute (4 h) rIL-2/endothelial interaction only and longer exposures (3–5 days) may result in cell death. Indirect cytolytic mechanisms cannot be ruled out (i.e. activation of other effector cells, release of secondary cytokines) [29, 35, 37].

Our observations on pulmonary artery endothelium may not reflect the interaction of rIL-2 and IALs with microvascular endothelium *in vivo*. However, rIL-2 and other cytokine receptors have been isolated from large vessel as well as microvascular endothelium [38–40], suggesting large vessel endothelium responds to IL-2 in a similar manner as microvascular endothelium. This similarity in response is supported by the parallel toxicity reported with cultured large vessel endothelium and *in vivo* model systems. Thus our findings reflect the *in vivo* rIL-2/lymphocyte/endothelial interactions.

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