on the intrinsic physiological pathways that normally operate when axons form synaptic contacts with target neurons or peripheral receptors.

These observations and others indicate that regenerating axons can be stopped by two different mechanisms—(i) by activating the physiological stop pathway that is built into the axon and (ii) by physically obstructing the advance of the axon tip (Fig. 2). In the adult mammalian spinal cord, both target neurons and astrocytes have the potential to stop axonal growth because of their capacity to activate the physiological stop pathway. Just as with target cells, astrocytes may contribute to the formation of neural connections by controlling the intrinsic tendency of axons to elongate. In the mature CNS, astrocytic processes envelop a substantial part of the surface of synaptic terminals; one of their roles may be to prevent axon terminals from wandering away from their synaptic contacts (*17*).

For effective regeneration in the CNS, axons must reconnect with their targets. The first and essential step in the sequence of reconnection is elongation of the axons. To promote axonal elongation within the substratum of the CNS, two different kinds of environmental factors must be considered. If the spinal cord of an adult mammal is transected, a connective tissue scar forms in the damaged region, and swollen axon terminals that are often filled with neurofilaments have been observed in contact with the scar (*9*). In this case, preventing or removing the connective tissue obstacle is clearly essential for axonal regeneration. This, however, may not be sufficient to promote axonal regeneration. In addition, the tendency of the mammalian astrocytes to limit the motility of the axon tip through the physiological stop pathway must also be considered.

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Eosinophils Cocultured with Endothelial Cells Have Increased Survival and Functional Properties

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Human peripheral blood eosinophils, cells often associated with allergic and parasitic diseases, were maintained in vitro for at least 14 days when they were cocultured with bovine endothelial cells and for at least 7 days when cultured with either bovine or human endothelial cell–derived conditioned medium. The cocultured eosinophils became hypodense and generated about three times as much leukotriene C_4 upon activation with calcium ionophore and killed about three times as many antibody-coated larvae of *Schistosoma mansoni* as freshly isolated normodense eosinophils. That these cells can be maintained in vitro by coculture with endothelial cells, and the surprising finding that the cocultured eosinophils have biochemical, cytotoxic, and density properties similar to those of eosinophils in patients with allergic and other disorders, will facilitate investigation of the regulation and role of these cells in health and disease.

EOSINOPHILS, WHICH ACCOUNT FOR 3 to 6% of the circulating granulocytes in humans, are thought to remain in the bloodstream for 6 to 12 hours before they enter connective tissues where they survive for several days (*1*). On the basis of density-gradient sedimentation, two populations of eosinophils (designated normodense and hypodense) have been identified (*2–4*). Hypodense eosinophils are prominent in the peripheral blood of patients with eosinophilia such as that associated with helminthic infections (*2–4*). Eosinophils are known to generate leukotriene C_4 (LTC_4) upon activation of the 5-lipoxygenase pathway (*5–7*) and to kill antibody-coated targets such as larvae of *Schistosoma mansoni* (*8, 9*). Both functions are enhanced in hypodense eosinophils (*4, 10*) and can be augmented in vitro in normodense eosinophils upon exposure to recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (*11*). We now report that eosinophils can be maintained in vitro by coculture with endothelial cells. The cocultured eosinophils produce more leukotrienes in response to calcium ionophore and kill more antibody-coated larvae of *S. mansoni* than freshly isolated cells.

When human peripheral blood eosinophils were cultured for 7 days at a density of 5.0×10^5 cells in 2 ml of enriched RPMI 1640, >99% ($n = 5$) of the cells died as assessed by uptake of Trypan blue. However, in a representative experiment when rep-

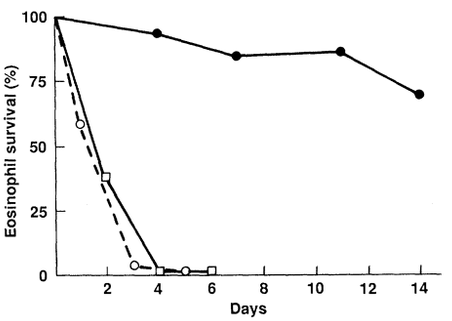

Fig. 1. The survival of eosinophils when cultured in enriched RPMI 1640 (○) or when cultured in enriched RPMI 1640 in the presence of endothelial cells (●) or 3T3 fibroblasts (□). Human eosinophils were isolated under sterile conditions to a purity of 88 ± 11% (mean ± SD, $n = 11$) according to a modification (*7, 29*) of the method of Vadas *et al.* (*9*), and residual contaminating erythrocytes were eliminated by hypotonic lysis. The results are typical of the eight and four experiments done on cells cultured for 7 and 14 days, respectively.

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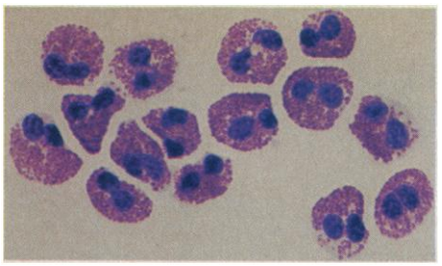

Fig. 2. Photomicrograph of eosinophils after 7 days of coculture with endothelial cells (magnification ×400). Cells were prepared by cytocentrifugation and stained with Wright's and Giemsa stains. Eosinophils represented >90% of the nonadherent cells in the culture.

licates of these isolated eosinophils were cocultured in the presence of about three times as many bovine pulmonary artery–derived endothelial cells, 85 and 69% of the eosinophils remained viable and nonadherent after 7 and 14 days, respectively (Fig. 1). In multiple experiments in which eosinophils were cocultured for 7 (n = 8) and 14 (n = 4) days, 85 ± 7% and 75 ± 12% (mean ± SD), respectively, of the original eosinophils remained viable. As assessed by Wright's and Giemsa staining, no mononuclear cells were present in the initial eosinophil preparations or in the cocultured cells. The cocultured eosinophils had large eosin-staining granules and had segmented nuclei (Fig. 2). Mitotic cells were not observed. Granule-containing eosinophils were distinguished from nonadherent endothelial cells by light microscopy (bright field) and represented >90% of the nonadherent cells in the coculture. As assessed by Trypan blue exclusion, >95% of the nonadherent endothelial cells were not viable. Thus, about 99% of the viable nonadherent cells were eosinophils.

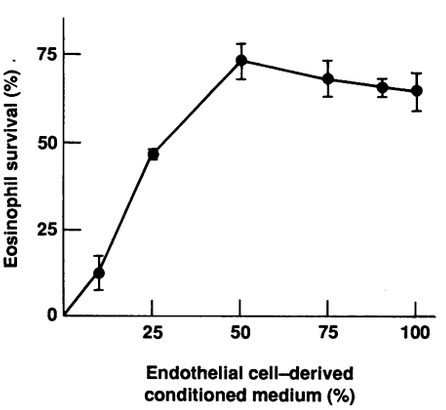

Fig. 3. The percentage of seeded eosinophils remaining viable after 7 days of culture with different concentrations of endothelial cell–derived conditioned medium. The average values plus or minus the range of duplicate cultures for a representative experiment are plotted. Conditioned medium was obtained from confluent bovine endothelial cells maintained for 48 hours in enriched RPMI 1640. The data are representative of four experiments.

The effect of endothelial cells on eosinophil viability appeared to be cell-specific, since neutrophils, the contaminating cell in the initial population of seeded leukocytes, were not present after 7 days of coculture. We further assessed the specificity of the cellular interactions by seeding 7.5×10^5 cells that contained about 95% neutrophils and about 5% eosinophils onto the endothelial cells. By day 4 of coculture, <2% of the neutrophils remained viable, whereas 90 ± 17% (mean ± SD, n = 3) of the seeded eosinophils in the same endothelial cell coculture were still viable. Replicate eosinophils were also cocultured with 3T3 fibroblasts derived from Swiss albino mouse skin, since these fibroblasts have been shown to maintain in vitro mast cells of various species (*12*). Whereas 79 ± 6% (mean ± SD, n = 3) of the eosinophils cocultured with endothelial cells remained viable at day 7, only 1 ± 1% of the eosinophils remained viable when cocultured with 3T3 fibroblasts.

To find out whether endothelial cells were secreting soluble factors capable of maintaining the viability of eosinophils in vitro, we cultured freshly isolated human eosinophils in the presence of 48-hour conditioned medium from cultures of bovine endothelial cells rather than in the presence of living endothelial cells. The number of surviving eosinophils increased as the concentration of conditioned medium increased from 0 to 50% (Fig. 3). The mean maximal survival of the seeded eosinophils after 7 days of culture was 73 ± 17% (mean ± SD, n = 4). The morphology of these cultured eosinophils was similar to that of the eosinophils cocultured with endothelial cells, and no other cell type was present.

We measured the functional capacity of the cultured eosinophils by stimulating them with calcium ionophore A23187 under conditions that were optimal for freshly isolated cells (*7*), and then by quantifying the generation of LTC_4 by radioimmunoassay (*13*). After 7 days of coculture with endothelial cells, the amount of LTC_4 generated by the eosinophils increased significantly compared to that from freshly isolated cells (Table 1). In other experiments in which eosinophils were activated before and after 7 days of culture in 50% endothelial cell–derived conditioned medium, 10^6 freshly isolated eosinophils and 10^6 cultured eosinophils generated 28 ± 11 and 65 ± 25 ng of LTC_4 (mean ± SD, n = 5), respectively ($P < 0.02$). The killing of antibody-coated larvae of *Schistosoma mansoni* by eosinophils (*8, 14*) was significantly enhanced after 7 days of coculture relative to that by freshly isolated cells (Table 2).

In experiments in which the density of eosinophils was compared before and after 7 days of coculture with bovine endothelial cells, the cocultured eosinophils were found to be less dense than the freshly isolated cells. The eosinophils were originally isolated from the normodense fractions 5, 6, and 7. As shown in Fig. 4, after 7 days of coculture none of the eosinophils sedimented at the original densities, but rather 9, 80, and 11% of the eosinophils sedimented in the hypodense fractions 1, 2, and 3, respectively. In four experiments, after 7 days of coculture, 99 ± 1% (mean ± SD) of the eosinophils were hypodense. In further experiments, the effect of culture in 50% bovine endothelial cell–derived conditioned medium on the density and the capacity to generate LTC_4 was assessed in a time-dependent fashion at three intervals over 7 days. In three experiments, 21 ± 9, 81 ± 9, and 96 ± 4% (mean ± SD) of the eosinophils shifted from normodense to hypodense at 1 hour, 1 to 2 days, and 7 days, respectively. In the same experiments, LTC_4 generation was increased after 1 hour of culture from 24 ± 9 (mean ± range, n = 2) to 76 ± 22 ng per 10^6 cells (mean ± SD, n = 3). This later level was not different from that generated on days 1 to 2 and day 7, for which the values were 67 ± 35 and 70 ± 16 ng per 10^6 cells (mean ± SD,

Table 1. Calcium ionophore–induced generation of LTC_4 by eosinophils. Duplicate samples of freshly isolated eosinophils or eosinophils cocultured for 7 days with endothelial cells were stimulated with 2.5 μM calcium ionophore A23187 (2×10^5 cells per 0.4 ml of Tyrode's buffer) for 30 minutes at 37°C in the presence of 20 mM L-serine. After the termination of the reactions by the addition of 2 ml of 4°C methanol, the methanolic extracts were processed and analyzed by radioimmunoassay for the total generated immunoreactive equivalents of LTC_4 (nanograms per 10^6 cells) (*7*).

Experiment	Generation of LTC_4 (nanograms per 10^6 cells) by	
	Fresh eosinophils	Eosinophils cocultured with endothelial cells
1a*	22	85
1b	13	31
2	25	37
3	25	89
4	14	37
Mean ± SD	20 ± 5	56 ± 26 ($P < 0.05$)†

*Experiments 1a and 1b were performed on different days with eosinophils from the same donor. †Statistical significance was determined by Student's paired t test.

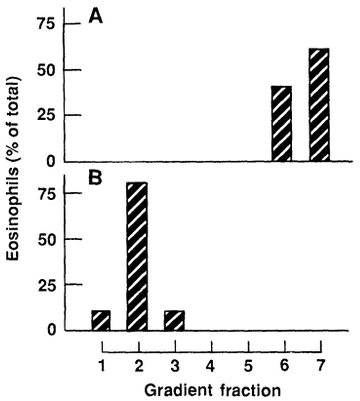

Fig. 4. The distribution of eosinophils on discontinuous metrizamide gradients before (**A**) and after (**B**) 7 days of coculture with endothelial cells. Fractions 1 to 6 refer to the cells at the 0/18, 18/20, 20/21, 21/22, 22/23, and 23/24% metrizamide interfaces. Fraction 7 refers to the combined 24% metrizamide layer and the cell pellet. The recovery of cocultured eosinophils after centrifugation was 90 ± 8% (mean ± SD, $n = 4$). The data are representative of four experiments.

$n = 3$), respectively. Thus, there was a dissociation between the time course for maximal LTC_4 generation and the change in density of the cultured eosinophils.

To assess whether soluble factors derived from human umbilical vein endothelial cells could also prolong the survival of human eosinophils in vitro, we cultured freshly isolated eosinophils in the presence of 48-hour conditioned medium from human endothelial cells (*15*). In three experiments, the survival of eosinophils was incrementally augmented when cultured with 1 to 25% conditioned medium. At the optimal concentration of 25% conditioned medium, the mean survival at 7 days was 67 ± 6% (mean ± SD, $n = 3$). In the same experiments, 10^6 freshly isolated eosinophils and 10^6 cultured eosinophils generated 37 ± 14 and 75 ± 28 ng of LTC_4 (mean ± SD, $n = 3$), respectively ($P < 0.05$). When the density of the eosinophils was compared before and after 7 days of culture in 25% human endothelial cell–derived conditioned medium, 97 ± 4% (mean ± SD, $n = 3$) of the viable cells were shifted from normodense to hypodense.

Table 2. Eosinophil cytotoxicity against larvae of *Schistosoma mansoni*. Triplicate samples of 100 antibody-coated larvae of *S. mansoni* were incubated for 24 hours at 37°C with 1×10^5 freshly isolated eosinophils or eosinophils that had been cocultured with endothelial cells for 7 days; the viability was assessed by microscopic analysis (*8, 14*).

Experiment	Percentage of larvae killed by	
	Fresh eosinophils	Eosinophils cocultured with endothelial cells
1a*	26	46
1b	7	21
2	15	34
3	5	33
Mean ± SD	13 ± 8	34 ± 9 ($P < 0.01$)†

*Experiments 1a and 1b were performed on different days with eosinophils from the same donor. †Statistical significance was determined by Student's paired t test.

Hypodense eosinophils have been found in the peripheral blood of patients with chronic helminthic infections (*2*), bronchial asthma (*4, 16*), atopy (*4*), the idiopathic hypereosinophilic syndrome (*17*), and neoplasia (*18*), as well as in the pleural lavage of patients with lung diseases (*3, 18*). Hypodense eosinophils have been reported to have enhanced cytotoxic (*4*) and chemotactic activity (*19*), enhanced generation of LTC_4 (*10*), and increased consumption of oxygen and glucose (*3, 4, 18*) compared to normodense eosinophils. That endothelial cells induced normodense eosinophils to become hypodense cells with increased functional capacity suggests that under certain conditions the hypodense phenotype may be a hyperactive postmitotic eosinophil rather than a less differentiated cell. The ability to induce normal eosinophils to change their phenotype in vitro to cells that resemble eosinophils found in a variety of disorders offers a unique opportunity to study the biology of these cells in disease.

Cultured bovine aorta–derived endothelial cells and cultured human umbilical vein–derived endothelial cells are known to secrete growth factors (*20*) that include platelet-derived growth factor–like molecules (*21, 22*) and molecules with colony-stimulating activity (*23*). Recombinant human GM-CSF has been reported to augment eosinophil function (*11, 24*) and to enhance the in vitro survival of these cells by 9 hours (*24, 25*) or more (*26*). Human endothelial cells produce this cytokine in response to stimulation with tumor necrosis factor (*27*) or interleukin-1 (*28*). The results from the current study suggest that endothelial cells may release cytokines in vivo that enhance the survival and immunologic function of eosinophils as they leave the circulation and enter an inflammatory site in tissue.

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29. Eosinophils were isolated from the peripheral blood of seven donors, none of whom were ingesting aspirin, nonsteroidal anti-inflammatory drugs, or corticosteroids. In five of the cell donors, <3% of the circulating leukocytes were eosinophils, whereas 10 to 15% of the leukocytes in two of the donors (who had allergic rhinitis and allergic conjunctivitis) were eosinophils. Eosinophils (5.0×10^5 to 7.5×10^5) in 2 ml of RPMI 1640 containing 10% (v/v) FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (10 μg/ml), 2 m*M* L-glutamine, and 0.1 m*M* nonessential amino acids (enriched RPMI 1640) were seeded into 35-mm culture dishes containing confluent endothelial cells (passages 19 to 29, CCL-209, American Type Culture Collection) prepared as previously described [P. J. Del Vecchio and J. R. Smith, *J. Cell. Physiol.* **108**, 337 (1981)], 3T3 fibroblasts (CCL-92, American Type Culture Collection), or enriched RPMI 1640 alone. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cell suspensions were removed every 48 hours. The nonadherent cells were sedimented at 250*g* for 10 minutes at 22°C, resuspended in fresh enriched RPMI 1640, and reapplied to the original plate. The percent survival of the cultured eosinophils was calculated as follows: 100 × (the total number of eosinophils excluding Trypan blue divided by the total number of eosinophils seeded). The calculated recoveries of the cultured eosinophils are minimum values because of cell losses during the media changes.

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