

Allergens, IgE, mediators, inflammatory mechanisms

Dexamethasone inhibits prolonged survival and autocrine granulocyte-macrophage colony-stimulating factor production by human eosinophils cultured on laminin or tissue fibronectin

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Background: Eosinophil autocrine generation of viability-promoting cytokines resulting from interaction with extracellular matrix (ECM) proteins may be important in promoting their persistence in allergic inflammation.

Objective: We examined eosinophil interaction with fibronectin, laminin, collagen, fibrinogen, hyaluronate, or vitronectin in terms of adhesion and for their ability to promote prolonged eosinophil survival by autocrine generation of IL-3, IL-5, or granulocyte-macrophage colony-stimulating factor (GM-CSF). The effect of dexamethasone was examined because it may inhibit integrin-dependent autocrine generation of viability-enhancing cytokines.

Methods: Microtiter wells were coated with different ECM proteins, and assays were performed to determine their levels of eosinophil adhesion, and tests were performed to determine their prolonged survival in culture. The receptors involved in these processes, the cytokines generated, and the effect of a dose range of dexamethasone were all examined. **Results:** Eosinophils cultured for 3 days on laminin or tissue fibronectin exhibited dose-dependent prolonged survival, which was not seen with any other ECM protein. Unstimulated eosinophils adhered to wells coated with laminin or fibronectin but not to wells coated with any of the other ECM proteins that were tested. Laminin-dependent eosinophil adhesion and prolonged survival were inhibited by specific monoclonal antibodies to $\beta 1$ and $\alpha 6 \beta 1$. Laminin-dependent survival was inhibited by a neutralizing antibody to GM-CSF, an anti-IL-5 antibody achieved a measurable but insignificant inhibition of survival, and an antibody to IL-3 had no effect. Increasing concentrations of dexamethasone (maximal at 10^{-7} mol/L) inhibited both laminin- and fibronectin-dependent enhanced eosinophil viability at 3 days. Eosinophils cultured on either laminin or fibronectin for 24 or 72 hours generated picogram quantities of GM-

CSF, which were inhibited by the addition of dexamethasone (10^{-7} mol/L).

Conclusion: We have confirmed that eosinophils adhere to laminin in an $\alpha 6 \beta 1$ -dependent manner, and this interaction results in the autocrine generation of GM-CSF, which enhances eosinophil survival in culture. Both laminin and tissue fibronectin-dependent autocrine GM-CSF generation were inhibited by physiologically relevant concentrations of dexamethasone. This may represent an important mechanism by which glucocorticoids reduce the tissue eosinophilia in allergic disease. (*J Allergy Clin Immunol* 1997;100:208-15.)

Key words: Eosinophil, dexamethasone, laminin, fibronectin, extracellular matrix, cytokines, integrins, prolonged survival

Infiltration of the tissues by often large numbers of eosinophils is one of the consistent features of asthma and related allergic diseases. The eosinophil's ability to generate an array of proinflammatory mediators has implicated it as a major effector cell in asthma pathogenesis.^{1,2} It is now widely accepted that adhesion to the blood vessel by proinflammatory cells represents an essential stage in eosinophil emigration into tissue, a process controlled by integrin receptors on leukocytes binding to counter-structures on the endothelial cell.^{3,4} The mechanisms responsible for selective eosinophil accumulation in the airways in asthma are complex but probably involve a combination of selective adhesion to bronchial postcapillary venular endothelium, followed by transmigration into the bronchial submucosa under the influence of chemotactic factors such as platelet activating factor, leukotriene B₄, and C-C chemokines such as RANTES and eotaxin.^{5,6}

Eosinophils function primarily in the tissues (there is about one blood eosinophil for every 100 tissue eosinophils),⁷ and it is here that they come into contact with the proteins of the extracellular matrix (ECM). A key feature of many of the proteins that make up the ECM is their ability to interact with cell-surface receptors on eosinophils and other proinflammatory cells with profound functional consequences. For example, eosinophils adhere to fibronectin in an $\alpha 4 \beta 1$ -dependent manner, a process that results in short-term priming of eosinophils for enhanced calcium ionophore-dependent

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Abbreviations used

BSA:	Bovine serum albumin
ECM:	Extracellular matrix
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
mAb:	Monoclonal antibody

leukotriene C_4 release⁸ or augmented eosinophil peroxidase release.⁹ However, ECM proteins can also modulate the response of eosinophils to physiologic soluble stimuli¹⁰; and platelet activating factor, C5a, or IL-5 stimulation of eosinophils adherent to human serum albumin resulted in eosinophil-derived neurotoxin release, which was inhibited if the cells were adherent to fibronectin or laminin.¹¹ In the longer term, $\alpha 4\beta 1$ -dependent adhesion to fibronectin prolonged eosinophil survival in culture due to autocrine production of IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5.^{12,13} Eosinophils have been shown to adhere to laminin in an $\alpha 6\beta 1$ -dependent manner.¹⁴ This finding was confirmed by Tourkin et al.¹⁵ who also demonstrated prolonged survival by eosinophils adherent to laminin as a result of $\alpha 6\beta 1$ -dependent autocrine cytokine production.

In this study we have confirmed that unstimulated eosinophils adhere to laminin or fibronectin and that laminin can also support eosinophil viability in culture by stimulating autocrine cytokine production. We have extended these observations by comparing both eosinophil adhesion and survival on tissue fibronectin or laminin with adhesion and survival on a range of ECM proteins. Glucocorticoids such as dexamethasone are well known for their ability to cause a striking reduction in eosinophil numbers *in vivo*.¹⁶ However, the precise mechanisms by which they exert their anti-eosinophilic effect remain obscure. One potential mechanism is the inhibition of autocrine generation of viability-promoting cytokines by eosinophils after integrin-dependent interaction with ECM proteins. We therefore also investigated the effect of dexamethasone on prolonged survival of eosinophils cultured on fibronectin or laminin.

METHODS

Reagents

Reagents were obtained as follows. Water soluble dexamethasone, endotoxin-free water, human tissue fibronectin, human laminin, collagen type IV, hyaluronate, fibrinogen, vitronectin bovine serum albumin (BSA; grade V), Histopaque 1083, rat serum, goat serum, and control mouse immunoglobulins were obtained from Sigma Chemical Co. (Poole, U.K.). Laminin was also purchased from Life Technologies Ltd. (Paisley, U.K.). Magnetic activated cell sorter columns and micromagnetic beads bound to anti-CD16 or anti-CD14 monoclonal antibody (mAb) were acquired from Eurogenetics Ltd. (London, U.K.).

Antibodies

Integrin $\beta 1$ mAb 4b4, $\alpha 6\beta 1$ mAb S341, and $\alpha 6\beta 1$ mAb AA3 were provided as part of the $\beta 1$ integrin subpanel by the 1993 International Workshop on Leukocyte Antigens. Polyclonal

antibodies against human GM-CSF or IL-3 were purchased from R&D Systems (Oxford, U.K.). The mAb to IL-5 (TRFK-5, a rat mAb to mouse IL-5 that cross-reacts with human IL-5) was a gift from Dr. Tim R. Mosmann (Edmonton, Alberta, Canada). All the mAbs were murine, and the anti-cytokine mAbs were selected for their ability to neutralize the bioreactivity of both their natural and recombinant cytokines. They showed no cross-reactivity with any other human cytokine.

Eosinophil purification

A volume of 100 ml of blood was obtained from normal donors or individuals with a history of mild allergic disease who were either free of symptoms or displayed mild allergic symptoms for which they were not taking medication at the time of venesection (eosinophilia $\leq 0.5 \times 10^6$ eosinophils/ml). Eosinophils were purified by negative immunomagnetic selection by using a modification of the method of Hansel et al.,¹⁷ which has been detailed elsewhere.¹³ Briefly, after dextran sedimentation to remove red blood cells, the leukocyte-rich supernatant was subjected to slow centrifugation to remove platelets. The leukocytes were then centrifuged on Histopaque 1083 cushions. The granulocyte pellet was transferred to a fresh tube and subjected to hypotonic shock with endotoxin-free water to remove any remaining erythrocytes. After incubation with micromagnetic beads coated with anti-CD16 and anti-CD14, the cells were passed through the magnetic activated separation column. By using this method, eosinophils with a purity greater than 99.9% (as judged by counting at least 1000 cells on a cyto-spin stained with May-Grunwald-Giemsa) were obtained. Preparations that were contaminated with more than 1 mononuclear cell per 1000 eosinophils were not used for these studies. Eosinophil viability was consistently greater than 99%, as judged by staining with trypan blue.

Adhesion assay

The adhesion assays were performed as previously described.¹⁸ Briefly, non-tissue-culture-grade 96-well plates (Beckton Dickinson, Oxford, U.K.) were coated with ECM proteins, and efficient coating was confirmed by ELISA.¹³ Before use, plates were washed, and nonspecific binding sites were blocked with 1% heat-inactivated BSA (heat-inactivated as previously described^{12,13}). Purified eosinophils were labeled with chromium 51, washed twice in medium 199 supplemented with 2% fetal calf serum to remove excess radioactivity, and adjusted to a concentration of 10^6 eosinophils/ml in the same medium. Cells were added to coated wells containing appropriate controls or mAb at a final concentration of 10^5 eosinophils/well. Preliminary experiments demonstrated maximal binding to both fibronectin and laminin at 30 minutes (data not shown). Plates were therefore incubated for 30 minutes at 37°C in all subsequent experiments. To remove nonadherent cells, each well was carefully filled with medium 199, the plate was sealed with acetate plate sealing tape and centrifuged upside down for 5 minutes at 50 g. The wells were carefully aspirated, and a visual check of each well was made to ensure uniform cell distribution throughout the wells. The cells were then lysed with 1% Triton X-100, aspirated, and washed twice to remove radioactivity. The supernatants were counted in a gamma counter, and the percentage of adherent cells was calculated by reference to the counts from 10^5 chromium 51-labeled eosinophils.

Eosinophil culture

Freshly purified eosinophils were washed with RPMI 1640 medium supplemented with 2% fetal calf serum, antibiotics,

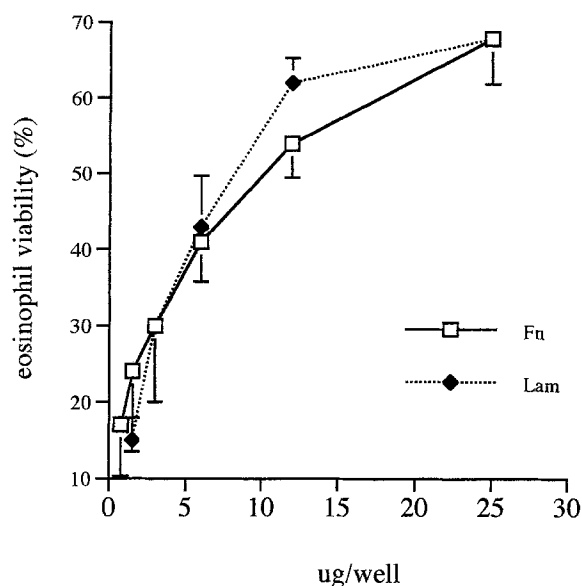


FIG. 1. Effect on eosinophil viability after 3 days in culture in microtiter wells coated with increasing concentrations of laminin (*Lam*) or tissue fibronectin (*Fn*). Eosinophil survival at 3 days in BSA-coated wells was $11\% \pm 3.3\%$. Each point represents mean $\pm$ SEM of three experiments.

and L-glutamine and adjusted to a concentration of $2 \times 10^6/\text{ml}$ in the same medium. Preliminary experiments established that this concentration of fetal calf serum produced optimal laminin or fibronectin-dependent eosinophil survival, but minimal survival on BSA (data not shown). Cells ($100 \mu\text{l}$) were cultured in 96-well tissue-culture-grade, flat-bottomed plates (Life Technologies) coated with the ECM protein of interest or BSA (heat-inactivated as previously described^{12,13}) at 37°C with 5% CO_2 in a humidified atmosphere. Plate coating was performed and validated as previously described.¹³ Before use the wells were washed and blocked with 1% heat-inactivated BSA for 60 minutes at 37°C . Before addition of the cells, each well was washed twice with RPMI, and the appropriate treatment or control was added. All mAbs were used at a final concentration of $5 \mu\text{g}/\text{ml}$. In the case of mAbs directed against eosinophil receptors, these were saturating concentrations as assessed by immunostaining and flow cytometry. Cytokine-neutralizing antibodies were added to give a final concentration of $10 \mu\text{g}/\text{ml}$ for each antibody. In some experiments, purified eosinophils were cultured in BSA-coated wells in the presence of GM-CSF at a final concentration of 10^{-12} mol/L with or without dexamethasone (10^{-7} mol/L).

Eosinophil viability was determined by addition of $50 \mu\text{l}$ of 1% trypan blue in phosphate-buffered saline to each well, followed by incubation for 5 minutes at room temperature. The well was carefully aspirated without disturbing any of the cells, and 0.1 ml of phosphate-buffered saline was added. The wells were then read in a blinded fashion with an inverted microscope, and the percentage of viable cells was determined. In some experiments, 1- or 3-day supernatants were removed immediately before addition of trypan blue and stored at -80°C for cytokine measurement (see below).

Cytokine assay

Separate experiments were performed in which supernatants from eosinophils cultured either on tissue fibronectin, laminin,

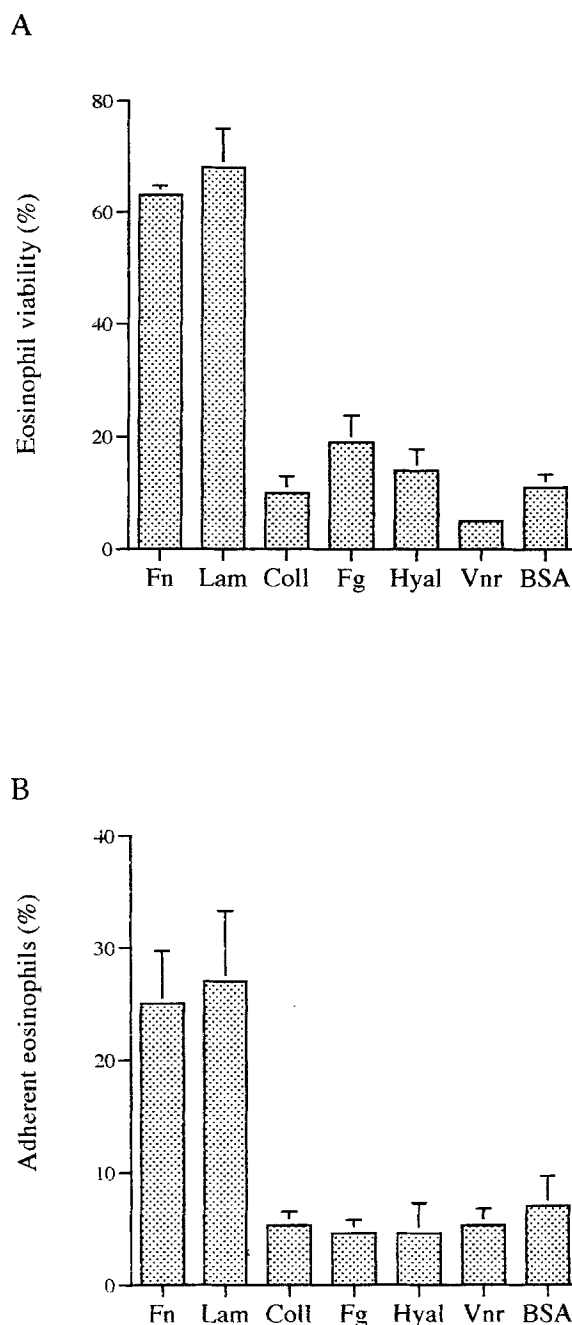


FIG. 2. **A**, Eosinophil viability after 3 days in culture on fibronectin (*Fn*, $25 \mu\text{g}/\text{well}$), laminin (*Lam*, $12.5 \mu\text{g}/\text{well}$), collagen IV (*Coll*, $50 \mu\text{g}/\text{well}$), fibrinogen (*Fg*, $50 \mu\text{g}/\text{well}$), hyaluronate (*Hyal*, $50 \mu\text{g}/\text{well}$), vitronectin (*Vnr*, $50 \mu\text{g}/\text{well}$), or BSA ($50 \mu\text{g}/\text{well}$). Each column represents mean $\pm$ SEM of three experiments. **B**, Unstimulated eosinophil adhesion to microtitre wells coated with fibronectin (*Fn*), laminin (*Lam*), collagen IV (*Coll*), fibrinogen (*Fg*), hyaluronate (*Hyal*, $50 \mu\text{g}/\text{well}$), vitronectin (*Vnr*), or BSA (at same concentrations per well as in **A**). Each column represents mean $\pm$ SEM of three experiments.

or BSA for 24 hours or on tissue fibronectin, laminin, ECM proteins, or BSA for 72 hours were removed and stored at -80°C until they were assayed. The viability of the eosinophils at these time points was also assessed by using trypan blue

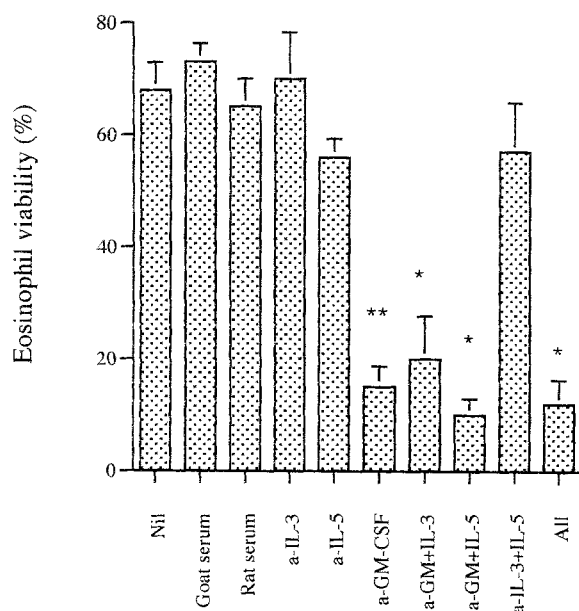


FIG. 3. Effect of neutralizing antibodies to IL-3, IL-5, or GM-CSF added alone or in combination on laminin-dependent eosinophil prolonged survival at 3 days. Each column represents mean $\pm$ SEM of five experiments. * $p < 0.05$; ** $p < 0.005$.

exclusion as described above. GM-CSF was measured by using a commercially available ELISA kit (Quantikine, R&D Systems). The assay was performed according to the manufacturer's instructions. The limit of assay sensitivity was 5 pg/ml, and the results are expressed in picograms per milliliter of GM-CSF generated by 2×10^5 eosinophils.

Statistical analysis

All data are derived from experiments performed in duplicate and are expressed as mean $\pm$ SEM. Significance tests were performed by using the Student's *t* test, and a *p* value of less than 0.05 was considered significant.

RESULTS

The viability of purified human eosinophils was enhanced in a dose-dependent fashion by culture in wells coated with increasing concentrations of tissue fibronectin or laminin. Optimal concentrations of laminin and fibronectin were 12.5 μ g/well and 25 μ g/well, respectively (Fig. 1). Increasing the coating concentration of each protein did not result in a measurable increase in eosinophil viability, and significant increases in viability with both proteins were observed for up to 4 days (data not shown). The viability of eosinophils cultured on laminin purchased from Sigma Chemical Co. was almost identical to that seen when eosinophils were cultured on laminin purchased from Life Technologies Ltd. (data not shown). Fig. 2, *A* illustrates eosinophil viability at 3 days in wells coated with ECM proteins. Only fibronectin- or laminin-coated wells supported eosinophil viability. Increasing the coating concentration of collagen IV, fibrinogen, hyaluronate, vitronectin, or BSA to 250 μ g/well did not result in any increase in eosinophil viability (data not shown).

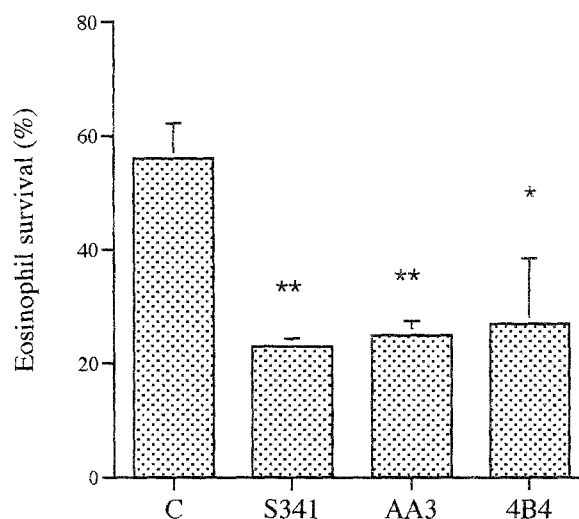


FIG. 4. The effect of mAbs to $\alpha 6\beta 1$ (S341 and AA3) and $\beta 1$ mAb (4B4) compared with an isotype-matched control mAb (C) on laminin-dependent eosinophil prolonged survival at 3 days. Each column represents mean $\pm$ SEM of four experiments. * $p < 0.025$; ** $p < 0.005$.

Eosinophil adhesion to wells coated with optimal concentrations of fibronectin or laminin was then compared with that of wells coated with the ECM proteins collagen IV, fibrinogen, hyaluronate, vitronectin, or BSA. Significant unstimulated eosinophil adhesion to both tissue fibronectin and laminin was observed, whereas no significant adherence to any of the other ECM proteins compared with BSA was seen (Fig. 2, *B*). Coating concentrations up to 250 μ g/well did not result in significant eosinophil adhesion to collagen, fibrinogen, hyaluronate, or vitronectin (data not shown).

We have previously demonstrated that tissue fibronectin-dependent enhancement of eosinophil viability in culture is due to $\alpha 4\beta 1$ -dependent autocrine production of IL-3, IL-5, or GM-CSF.^{12, 13} We therefore examined whether the observed survival on laminin-coated wells was a result of autocrine generation of viability-promoting cytokines. Fig. 3 illustrates the survival of eosinophils cultured for 3 days on laminin in the presence of neutralizing mAbs to IL-3, IL-5, or GM-CSF added alone or in combination. The relevant animal sera (goat and rat) were also included as control samples. A small but nonsignificant (compared with the viability of eosinophils cultured on laminin in the presence of rat serum) decrease in viability was observed in the presence of neutralizing antibodies to IL-5, whereas anti-IL-3 had no measurable effect. In contrast, an antibody to GM-CSF caused a significant ($p < 0.005$) fall in eosinophil viability. Experiments performed with combinations of the anti-cytokine antibodies did not demonstrate any significant additive effect of the anti-IL-3 or anti-IL-5 antibodies when added together, when combined with the anti-GM-CSF, or when all three antibodies were used together. We next investigated the role of the integrins $\beta 1$ and $\alpha 6\beta 1$ in laminin-dependent eosinophil adhesion

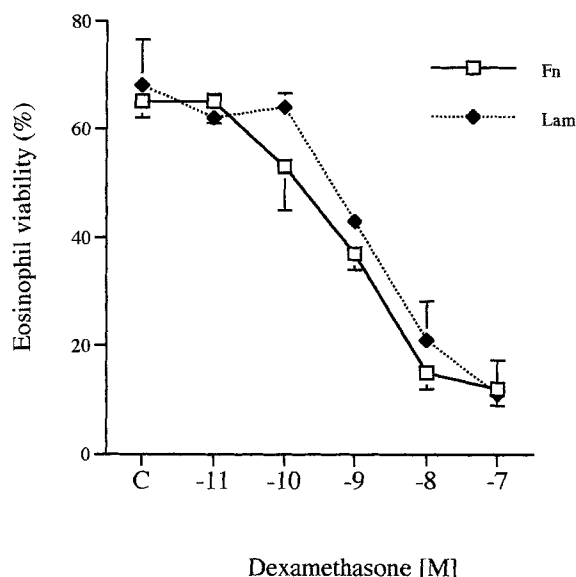


FIG. 5. Inhibitory effect of increasing concentrations of dexamethasone on laminin (Lam)- or tissue fibronectin (Fn)-dependent prolonged eosinophil survival at 3 days. Each point represents mean $\pm$ SEM of five experiments.

and survival. As reported,^{14,15} mAbs to $\alpha 6\beta 1$ (S341 and AA3) were found to inhibit eosinophil adhesion to laminin (data not shown). We observed significant inhibition of eosinophil viability on laminin-coated wells by mAbs to both $\alpha 6\beta 1$ and $\beta 1$, but not by an isotype-matched control mAb (Fig. 4).

The effect of increasing concentrations of dexamethasone on both fibronectin- and laminin-dependent eosinophil survival was examined next. Dexamethasone was found to inhibit the viability-promoting effects of both fibronectin and laminin in a dose-dependent manner. Maximal effect in both cases was seen at a concentration of 10^{-7} mol/L with a median effective concentration of 10^{-9} mol/L (Fig. 5). These data were confirmed by measuring GM-CSF production by eosinophils adherent to fibronectin or laminin by using a specific ELISA. Supernatants from eosinophils cultured for 24 or 72 hours with fibronectin or laminin were found to contain picogram quantities of GM-CSF. No measurable concentrations of GM-CSF were detected in wells coated with any other ECM protein (data not shown). Culture of eosinophils with dexamethasone at a concentration of 10^{-7} mol/L in wells coated with optimum concentrations of laminin or fibronectin resulted in significant inhibition of autocrine GM-CSF production at both 24 and 72 hours (Fig. 6). The viability of eosinophils at 24 hours was $89\% \pm 3.1\%$ for laminin-coated wells and $86\% \pm 3.2\%$ for fibronectin-coated wells, and in the presence of dexamethasone the viabilities of eosinophils were $88\% \pm 3.2\%$ and $86\% \pm 1.3\%$, respectively. Autocrine GM-CSF production by eosinophils cultured in wells coated with fibronectin or laminin was also abolished by anti- $\beta 1$ or $\alpha 6\beta 1$ mAb (data not shown).

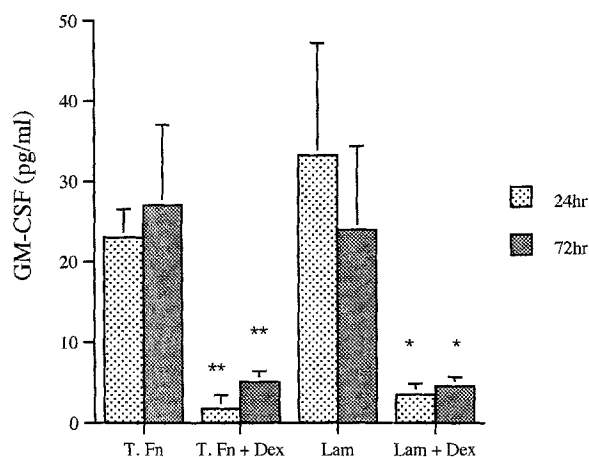


FIG. 6. Inhibitory effect of dexamethasone on laminin (Lam)- and tissue fibronectin (Fn)-dependent autocrine GM-CSF generation by eosinophils cultured for 24 or 72 hours. Eosinophil viability at 24 hours on fibronectin was $86\% \pm 1.3\%$ and $86\% \pm 3.1\%$ in the presence and absence of dexamethasone, respectively. Eosinophil viability on laminin at 24 hours was $88\% \pm 3.2\%$ and $89\% \pm 3.1\%$ in the presence and absence of dexamethasone, respectively. Each column represents mean $\pm$ SEM of four experiments. * $p < 0.05$; ** $p < 0.025$.

Finally, we investigated the effect of dexamethasone on the viability of eosinophils cultured with GM-CSF at a concentration of 10^{-12} mol/L, chosen because it corresponds to the quantity of cytokines detected in supernatants of eosinophils cultured on laminin or tissue fibronectin for 3 days. Although dexamethasone abolished autocrine GM-CSF production by eosinophils adherent to laminin or fibronectin at a concentration of 10^{-7} mol/L, the same dexamethasone concentration had no effect on viability of eosinophils when cultured for 3 days with GM-CSF compared with cells cultured with GM-CSF alone (Table I). Anti- $\beta 1$ or $\alpha 6\beta 1$ mAb had no significant effect on viability of eosinophils cultured for 3 days with GM-CSF (data not shown).

DISCUSSION

We have previously shown that eosinophils cultured on fibronectin exhibit prolonged survival in culture as a result of $\alpha 4\beta 1$ -dependent autocrine generation of IL-3 and GM-CSF.¹² Recently, these findings were extended to demonstrate enhanced eosinophil survival on tissue fibronectin compared with that on plasma fibronectin.¹³ This finding was consistent with the observation that tissue fibronectin has the alternatively spliced IIICS region, which contains the CS-1 and CS-5 sequences recognized by $\alpha 4\beta 1$, in both of its subunits whereas plasma fibronectin lacks it in at least half of its subunits.¹⁹ In this study we have confirmed the observation that human eosinophils adhere to laminin through the integrin $\alpha 6\beta 1$.¹⁴ We have further confirmed a previous report¹⁵ that eosinophils cultured on microtiter wells coated with laminin will produce viability-enhancing cytokines in an autocrine fashion and that this interac-

tion appeared to be $\alpha\beta 1$ -dependent, because generation of GM-CSF in the supernatants of eosinophils cultured on laminin was blocked by anti- $\alpha\beta 1$ mAb. This inhibitory effect might be a consequence of inhibition of eosinophil adhesion to laminin, but other modulating effects of the mAb on eosinophil function cannot be ruled out. We have further extended these observations to show that eosinophil survival was not enhanced by culture on ECM proteins to which they did not adhere (i.e., collagen IV, fibrinogen, hyaluronate, vitronectin, or BSA) compared with eosinophil viability in excess of 60% at 3 days in wells coated with fibronectin or laminin. Using antibodies that have been shown to neutralize the viability-promoting effects of IL-3, IL-5 or GM-CSF,¹³ we were able to demonstrate that laminin-dependent prolonged eosinophil survival appeared to be mainly a consequence of autocrine generation of GM-CSF. The small but nonsignificant effect of the anti-IL-5 mAb suggested some contribution to prolonged survival by this cytokine, whereas IL-3 did not appear to be produced by eosinophils cultured on laminin in quantities sufficient to contribute to the observed prolonged survival. Moreover, experiments with combinations of the anti-cytokine antibody provide additional evidence that eosinophil culture on laminin-coated wells resulted mainly in the generation of GM-CSF as the major viability-enhancing cytokine. In contrast, we have previously demonstrated that eosinophils cultured on tissue fibronectin produced significant quantities of IL-3 and IL-5, as well as GM-CSF, in an autocrine fashion.¹³ These data suggest that the profile of viability-enhancing autocrine cytokine production by eosinophils may be profoundly influenced by the nature of the ECM substrates to which they are adherent and that their prolonged survival may be due to triggering through multiple integrin receptors.

There are a number of discrepancies between the results presented here and those reported by Tourkin et al.¹⁵ That study showed low (20%) but significant prolonged survival by eosinophils cultured on collagen IV and also demonstrated partial inhibition of laminin-dependent eosinophil viability by an anti-IL-3 antibody. A possible explanation for these variations in results is that the eosinophil preparations used by Tourkin et al.¹⁵ were contaminated with between 1% and 9% mononuclear cells. We have recently shown that the presence of mononuclear cell contamination of less than 1% can confound studies designed to assess eosinophil prolonged survival on ECM proteins because of integrin-dependent autocrine generation of significant quantities of viability-enhancing cytokines by the contaminating mononuclear cells.¹³ Thus this study confirms and extends the findings of Tourkin et al.,¹⁵ but, importantly, it also excludes the possibility that contaminating mononuclear cells might be contributing to the observed cytokine production by eosinophils cultured on laminin-coated wells.

Unstimulated eosinophil adhesion to laminin was found to be $\alpha\beta 1$ -dependent, and both here and in a

TABLE 1. Percent viability of eosinophils cultured for 3 days

	mean $\pm$ SEM			
Nil (%)	12	10	5	9 $\pm$ 2.1
GM-CSF (%)	85	76	75	78 $\pm$ 3.2
GM-CSF + dexamethasone (10^{-7} mol/L) (%)	80	89	70	80 $\pm$ 5.5

GM-CSF was added to give a final concentration of 10^{-12} mol/L.
Nil, No GM-CSF.

separate study,¹⁸ we demonstrated significant $\alpha\beta 1$ -dependent adhesion by unstimulated eosinophils to tissue fibronectin. The effect of stimulation on eosinophil adhesion was not assessed in this study, because we wished to compare the relationship between eosinophil prolonged survival on ECM proteins with their ability to adhere to those proteins. We have demonstrated that unstimulated eosinophils did not adhere to fibrinogen, collagen IV, hyaluronate, vitronectin, or BSA compared with significant adhesion to tissue fibronectin or laminin. In contrast, other reports have shown only negligible adherence by unstimulated eosinophils to fibronectin or laminin. Prior eosinophil activation with platelet activating factor¹¹ or a $\beta 1$ integrin-activating antibody²⁰ was required before significant adhesion to these ECM proteins was observed. There are a number of possible explanations for these discrepancies. One is the source of the eosinophils used by different groups. For example, the study by Kita et al.¹¹ used eosinophils isolated from nonallergic donors, whereas in this study eosinophils were purified from individuals with a history of mild allergic symptoms, who were not taking any medication and who had a mild eosinophilia not greater than 0.5×10^6 eosinophils/ml. Thus in vivo activation of eosinophils may contribute to their ability to adhere to matrix proteins. Another complication is that eosinophils also express Mac-1 (CD11b), which is a promiscuous receptor with a number of ligands. Engagement of this receptor has been shown to affect eosinophil function,²¹ and this may provide a partial explanation for the conflicting data from different groups. Additionally, both fibronectin and laminin exist in a number of different forms depending on their cellular source. Fibronectin is encoded by a single gene, but alternative splicing of the primary RNA transcript results in polypeptide diversity, which appears to be regulated in a cell type-specific fashion.^{22,23} Cellular interactions with laminin are complicated by the fact that it has at least six short peptide integrin recognition sites in the three subunits for which the expression varies among the different laminin isoforms. Thus the laminins represent a complex group of molecules that have a host of mechanisms for cellular interaction.²⁴ Differing results from studies performed by separate groups might be explained, at least in part, by the source of their ECM proteins, although we found no measurable differences in eosinophil prolonged sur-

vival when the cells were cultured on laminin from two different manufacturers.

Eosinophil prolonged survival on both fibronectin and laminin was inhibited by dexamethasone at low and physiologically relevant concentrations. We used a sensitive ELISA technique to demonstrate that supernatants from eosinophils cultured for 1 or 3 days on fibronectin or laminin contained picogram quantities of GM-CSF. Addition of dexamethasone was found to inhibit autocrine generation of GM-CSF by cells adherent to laminin or fibronectin for 24 or 72 hours. Furthermore, dexamethasone also inhibited GM-CSF generation by eosinophils with greater than 85% viability, cultured for 24 hours on laminin or fibronectin. This suggests that inhibition of survival was due to dexamethasone inhibiting ECM-dependent GM-CSF generation rather than diminished eosinophil survival resulting in decreased cytokine production. Consistent with this conclusion was the observation that dexamethasone had no effect on the viability of eosinophils cultured for 3 days in a concentration of exogenously added GM-CSF comparable to that generated by eosinophils adherent to laminin or fibronectin. This observation is in agreement with previous reports that the inhibitory effect of steroids on cytokine-induced prolonged survival is only seen at low cytokine concentrations and can be overcome if their concentration is increased.²⁵⁻²⁷ There is evidence both from in vitro studies^{25, 28} and from a model that used parasitized rats²⁹ that dexamethasone might act by inducing apoptosis or programmed cell death in eosinophils. However, in the study by Meagher et al.²⁸ this effect did not appear to be a result of inhibition of eosinophil cytokine generation. In this study we did not examine the apoptosis-inducing effects of dexamethasone on eosinophils cultured on laminin or fibronectin. Furthermore, dexamethasone might have an inhibitory effect on autocrine cytokine transcription, translation, secretion, or a combination of these factors. The precise mechanism by which dexamethasone inhibits laminin- or fibronectin-dependent autocrine production of cytokines by eosinophils is under investigation and will be the subject of a future report.

In conclusion, we have confirmed previous observations that in the short term, human eosinophils adhere to laminin and fibronectin through $\alpha\beta 1$ and $\alpha 4\beta 1$, respectively. No significant adhesion to any other ECM protein was observed. Long-term culture of eosinophils on laminin enhanced eosinophil survival as a result of autocrine generation of GM-CSF, which was not dependent on the presence of contaminating mononuclear cells. Physiologically relevant concentrations of dexamethasone inhibited both fibronectin- and laminin-dependent autocrine production of GM-CSF, even when eosinophil viability was high. This may represent an important mechanism by which dexamethasone can reduce tissue eosinophil numbers by inhibiting laminin- or fibronectin-dependent autocrine production of viability-enhancing cytokines.

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