T-LYMPHOCYTES AND CYTOKINES IN BRONCHOALVEOLAR LAVAGE IN ASTHMATIC PATIENTS

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INTRODUCTION

Over the past ten years a series of bronchoscopic studies have rádically altered our understanding of the pathophysiological processes involved in asthrna. Even in patients with mild asthma, bronchial biopsies show inflammatory changes, with fragility of the bronchial epithelium and subepithelial fibrosis, and a mixed cellular infiltrate. Light and electron microscopy reveal degranulation of mast cells and eosinophils and there is in addition evidence of activated mononuelear cells. These findings generated the concept of asthr:na as an inflammatory condition and have led to a search for the cells and molecules which might control the inflammatory process. Bronchoalveolar lavage has been a vital tool in this search, permitting access to the asthmatic airways in life and complementing the biopsy material which can only be obtained from the more proximal airways. BAL studies in asthma have focused on three important areas: (i) the cytokines which are thought to control IgE production and eosinophilia; (ii) the cytokines and chemokines implicated in cellular recruitment and activation; (iii) the properties of the T-lymphocytes which are present in the asthmatic airway. These have each been studied in stable asthma and following allergen provocation.

Much attention has been paid to the Th2 cytokine complex on chromosome 5q3l-33. This region contains the genes for IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF as weIl as the gene for the B-chain of the Fc receptor[(1)]. IL-4 plays a critical role in IgE regulation: IgE production is Tcell dependent but before switching over to make IgE the B-cell requires three distinct signals. Firstly there is the recognition of antigen by the B-cell antigen receptor (surface lg); second a soluble (cytokine) signal which is usually IL-4, but IL-13 can substitute and thirdly, a contact signal mediated throiugh CD40 and its ligand. Eosinophil growth and differentiation are controlled by the cytokines interleukin 3, interleulcin 5 and GM-CSF. These cytokines are principally produced by lymphocytes but mRNA for GMCSI can also be indentified in macrophages and bronchial epithelial cells. Co-labelling studies have demonstrated that the mRNA hybridisation signals for IL-3, IL-5 and GM-CSF in bronchial lavage cells are confined to T-cells[(2)]. Consequently, there has been considerable interest in the role of T-lymphocytes in atopic allergy.

Interestingly, when bronchial biopsies are stained for IL-4, the overwhelming majority of immunoreactive IL-4 is found in mast cells and little or any can be seen in T-cells. The lack of T-cell staining is most likely due to the limited storage capacity of the lymphocyte, but another important conclusion is that mast cells themselves may contribute to the enhanced IgE production seen in atopie individuals, by releasing IL-4 into the local milieu and favouring the differentiation of T-cells which in turn favour switching to IgE[(3)].

CYTOKINES IN LAVAGE FLUID INCLUDING CHEMOKINES

A wide range of cytokines have been sought in lavage fluid and in supernatants of stimulated and unstimulated BAL leukocytes. Increased amounts of TNF- and IFN- are released by BAL cells from asthrnatic airwaysl while after inhalation challenge production of both TNF- and IL-6 is increased[(5)]. Other studies have demonstrated increased BAL concentrations of IL-IB, GM-CSF, IL-2, IL-6 and TNF in symptornatic asthrnatic airwayS6. Interestingly, IL-1B

concentrations were found to be much higher at 4 am than at 4 pm, suggesting that this cytokine may play a role in the characteristic noctumal exacerbation of symptoms[7].

There has been particular interest in the role of chemotatic cytokines (chemokines) in recruiting inflanunatory leukocytes such as neutrophils and eosinophils into the airways. We have studied the role of interleukin-8 in bronchial asthrna and found that while there was little elevation in stable asthrna, allergen challenge induced quite marked elevations in IL-8, which appeared to be coming from the bronchial epithelium[8]. Another group, of cytokines under current scrutiny are the cytokines which have been implicated in fibrogenesis. Collagen deposition beneath the bronchial epithelial basement membrane is a characteristic feature of the asthmatic airway. In cohort studies and challenge studies, we have shown that BAL fluid from, asthmatic airways contains elevated concentrations of basic fibrogenic growth factor (bFGF) and TGF8. BFGF is found in increased amount 10 minutes after local allergen challenge and appears to be released from mast cells, whereas TGF-B co-localises to eosinophils and only increases 6 hours after local allergen challenge suggesting these two fibrogenic cytokines have distinct cells sources and kinetics in the process of airways inflammation [9].

T-CELL PHENOTYPES IN BAL INCLUDING TCR DATA

T-cell content of asthmatle airways

As discussed above, T-lymphocytes are intimately involved in the generation of allergic responses, through their ability to, recognise antigens, become activated and produce cytokines which stimulate surrounding cells. The T-lymphtocyte content of the, asthmatic airway is slighT_1y increased compared to control subjects but the principal difference is that the T lymphocytes present in asthmatic biopsies are activated as shown by expression of the interleukin 2 receptor (CD25) and the presence of mRNA for the cytokines interleukin-5 and granulocyte-macrophage colony stimulating factor. These changes are clinically relevant in that there is an association between eosinophil infiltration, T cell activation, and the degree of bronchial hyperresponsiveness. The forces that shape the airways Tcell repertoire remain obscure, and crucial questions remain unanswered regarding the antigen-specificity of the airways Tcells and the significance of the expression of activation markers.

Attempts to, establish the frequeney of allergen-specific T-cells by cloning T-cells from the human airway have, proved extremely difficult. Best current estimates are that at most 1 in 500 and perhaps less than 1 in 2,500 airways T-cells recognise house dust mite[10-11]. Recognising the difficulty in growing allergen-specific clones, we have assessed the airways T-cell repertoire by studying the usage of T-cell receptor (TcR) V-B genes in peripheral blood and BAL. Tcells were studied by 3-colour flow cytornetry using a panel of monoclonal antibodies to 16 TcR-VB families, and appropriate surface markers. All 16 VB families were present in varying proportions in the normal and asthmatic subjects studied. In all subjects, TcR usage in BAL CD8+ T-cells was similar to that in peripheral blood. In BAL CD4+ Tcells, there was over-representation of VB7.1 in most of the nonnal and asthmatic subjects.- In the normal subjects the, other TcR VB families were expressed in proportion to the, peripheral blood usage. In the asthrnatic subjects a number of additional TcR VB families were also expanded. PCR analysis and sequencing of TcR mRNA from two of the asthmatie BALs has revealed that the over-representation of VB7.1 was due to polyclonal expansion but BAL Tcells expressing VB4 were oligoclonal. These data indicate that VB7.1 may be a marker of airways-specific T-cell subset while the other expanded TcR families are consistent with either a superantigenic stimulus or autoimmune process[12].

Functional properties of BAL T-cells

To investigate the T-cell cytokine response when asthmatic airways are exposed to allergen, we obtained BAL T-cells 10 minutes or 24 hrs after local endobronchial allergen challenge in atopic asthrnatic subjects. After washing

and enriching for T-cells by adherence to plastic, BAL T-cells were cultured overnight with medium or PHA 2ug/ml, the cells were collected and total RNA was extracted for RT-PCR to assess expression of ten cytokines (TNF, IFN, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13 and GM-CSF). The unstimulated 10 minute polycIonal Tcell populations expressed mRNA for IFN-, IL-13 and TNF-. MRNA for IL-4 was detected in 1/6 samples and IL-5 mRNA in 2/6 but IL-3 mRNA was not detected. Overnight stimulation in vitro with PHA increased of IL3, IL-4 and IL-5 mRNA in 4/6 samples. 24 hrs after allergen challenge, mRNA for IL-13 and GM-CSF was readily detectable in unstimulated cells from sites challenged with saline or allergen but there was less mRNA for IFNthan in the 10 minute samples. After PHA stimulation in vitro, IL-4 and IL-3 were strongly upregulated while lesser amounts of mRNA for TNF and IFN were detectable, compared to the 10 minute samples. Cells from allergenchallenged sites showed weaker cytokine mRNA signals than cells from saline-challenged sites. BAL lymphocytes obtained immediately after allergen or saline challenge showed spontaneous expression of mRNA for IFN-, IL13 and TNF- The cytokines IL-4 and IL-5 could be induced by PHA stimulation in only a minority of samples. In contrast, BAL T-cells obtained 24 hours after allergen challenge expressed mRNA for a greater variety of cytokines including IL-4, IL-13, GM-CSF and IFN- [(13)].

Only a limited number of studies have addressed cytokine production by BAL cells before and after allergen challenge. Virchow et a. found increased concentrations of IL-1, IL-2, IL-5, IL-6, IL-8 and TNF- but no increase in IL4 or IFN- in lavage fluid 18 hours after challengel[(14)]. Zangrilli et al. have reported increased concentrations of IL-4 and IL-5 in concentrated BAL fluid 24 h after local allergen challenge, which was also associated with eosinophil influx and the release of soluble VCAM-1[(5)]. In contrast, Huang et al. found marked increases in IL- 13 with relatívely little IL-4 or IL5 protein 18-24 h after segmental allergen challenge, and identified the cellular source of the IL-13 as the mononuclear cell fraction[(16)]. Taken together, these studies suggest that EL-13 may be quanútatively more important than IL-4 in the local regulation of IgE switching in astítmatic airways.

T-CELL CLONING

To address the heterogeneity of these responses, T-cell clones were derived from BAL by a direct limiting dilution technique. BAL T-cells were cloned by limiting in the presence of PHA 2ug/ml and irradiated autologous PBMC. The clones were fed with 50 U/ml IL-2 on day 5 and examined for positive growth on day 14. Wells in wihch Poissonian statistics indicated a high > 85% probability of monoclonality were expanded with PHA, IL-2 and irradiated PBMC. After 6-8 weeks the clones were phenotyped by flow cytometry and cytokine profiles were obtained by RT-PCR. The proportion of BAL T-cells that could be cloned with PHA was about 1/50 for both the allergen - and saline-challenged sites (Fig. 3). There was no difference in the frequency of obtaining and five of these panel were cultured successfully to 8 weeks for full analysis. A panel of clones derived 4h after saline challenge showed strong mRNA signals for IL-13 with co-expression of IL-4 and IFN- consistent with a Th0 profile, whereas clones derived 24h after allergen or saline challenge expressed IL-13, GM-CSF, IL3, IL-4, and often IL-5, a pattem closer to the Th2 profile. There was considerable heterogeneity in the pattems of cytokine mRNA and protein production by the different clones, with no clear concordance between mRNA intensity and the amount of IL-4 or IFN- released. These data are broadly consistent with the view that airways T-cells produce pro-allergic cytokines, but emphasise the need to analyse both mRNA and protein to obtain an accurate picture of the cytokine profiles of airways T-cells.

INTRACELLULAR STAINING CYTOKINES

Recognising that T-cell cloning only permits the analysis of a small proportion of airway T-cells, we have applied a novel cytometric technique of intracellular cytokine staining to assess cytokine production within 6 hoursí of performing BAL. The method uses phorbol myristic acetate and ionomycin to stimulate cytokine production and the fungal metabolite monensin to paralyse the Golgi apparatus of the cells and prevent export of any cytokines produced.

After 5 hours incubation the cells are fixed, permeabilized and stained for cytokines as well as for surface makers before analysis on a flow cytometer. Using this technique, we found that the major difference between BAL Tcells from asthmatic airways and cells from nonasthmatic subjects was the increased proportion of BAL T-cells from asthmatic airways which produced IFN- alone[17]. A relatively small proportion of cells stained for IL-4, even when stimuli thought to favour IL-4 production were employed.

CONCLUSIONS

Analysis of TcR repertoires indicates that BAL T-cells are a selected population compared to peripheral blood. When individual BAL T-cells were cloned by limiting dilution and subcultured, a considerable degree of functional heterogeneity was observed, with most clones showing a Th2-typC pattem of cytokine mRNA expression. Interestingly, there were several hundred-fold differences in cytokine protein production between BAT T-cell clones with similar mRNA profiles. These results emphasise the importance of using several tec1miques to address the question of cytokine production by human airways cells and particularly the need to study protein and mRNA together to arrive at useful and valid conclusions.

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