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Experimental hypersensitivity pneumonitis: influence of Th2 bias

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Summary. Cultured murine CD4+ cells from *Saccharopolyspora rectivirgula* sensitized C3H/HeJ (Th1 bias) donors can adoptively transfer murine experimental hypersensitivity pneumonitis (EHP). We sensitized BALB/c mice (Th2 bias) with *S. rectivirgula*, obtained spleen and lung associated lymph node (LALN) cells, cultured the cells with specific antigen, and attempted adoptive transfer of EHP. We also treated both C3H/HeJ and BALB/c donor mice with IL4 and anti-IFNγ before exposure to *S. rectivirgula* and then cultured cells from both spleen and LALN before attempted transfer of EHP. We found that cultured spleen and lung associated lymph node cells can adoptively transfer EHP in both C3H/HeJ and BALB/c mice as demonstrated by infiltration of the recipient lungs with CD4+ lymphocytes. Treatment of both mouse strains with IL4 and anti-IFNγ did not change the ability of cultured cells to adoptively transfer EHP. We conclude that EHP induced by *S. rectivirgula* can occur in animals with either a Th1 or a Th2 bias and is not altered by treatment with IL4 and anti-IFNγ. This suggests that attributes of the antigen and not genetic background or cytokine environment at the site of initial sensitization determines the results of exposure to *S. rectivirgula*.

Keywords: hypersensitivity pneumonitis, IL4

Hypersensitivity pneumonitis (HP) is a group of lung diseases that result from repeated pulmonary exposure to various organic, antigenic materials with uncertain immunopathogenetic mechanisms. Despite apparent uniform exposure, most subjects exposed to the agents that cause HP do not develop clinically evident disease in mice (Schuyler 1997).

The relative importance of genetic background, nature of the immune response and antigen attributes in expression of HP are not known. Although early studies suggested an association of specific HLA haplotypes with susceptibility to development of HP, most recent reports do not support this hypothesis. The most consistent association between development of HP and the immune response is the protective effect of current cigarette smoking against expression of HP (Schuyler 1997). Cigarette smoking has many effects on the immune response, in general acting as a mild immunosuppressive agent.

Our model of adoptive transfer of experimental hypersensitivity pneumonitis (EHP, using *S. rectivirgula*, one agent that causes Farmer's Lung Disease in humans; Schuyler 1997), in mice (Schuyler *et al*. 1991) is useful for distinguishing events that lead to development of

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effector cells from events that occur after interaction of effector cells and antigen. Murine adoptive EHP is mediated by appropriately cultured CD4+ cells (Schuyler *et al*. 1992), and CD4+ cell lines derived from lung associated lymph nodes can be established that either have the characteristics of Th1 cells (high IFNγ and low IL4 secretion) which transfer EHP, or Th2 cells (low IFNγ and high IL4) which do not transfer EHP (Schuyler *et al*. 1997).

BALB/c mice produce a Th2 type response in various murine models of infectious and autoimmune diseases (Hsieh *et al*. 1995; Pearlman *et al*. 1995; Reiner & Locksley 1995; Urban *et al*. 1996). Since all the previously tested mouse strains in experimental hypersensitivity pneumonitis (C3H/HeJ, SJL/J and C57Bl/6) (Schuyler *et al*. 1991) exhibit a Th1 bias, we tested the hypothesis that BALB/c mice would respond in a manner different than these strains.

We also attempted to change the nature of the immune response to *S. rectivirgula* in both C3H/HeJ and the BALB/c mice by administration of IL4 and antibody to IFNγ, methods that have been used to change a Th1 to a Th2 response and alter the response to infectious challenges in other murine models. We examined specific antibody isotype response to *S. rectivirgula*; and cytokine secretion and total immunoglobulin isotype in cultured cells from both C3H/HeJ and BALB/c mice, as these have been found to reflect the nature of the immune response and to change as a result of experimental manipulation.

We found qualitative, but not quantitative differences between C3H/HeJ and BALB/c mice in ability to adoptively transfer EHP. Treatment of both C3H/HeJ and BALB/c animals with IL4 and anti-IFNγ did not change the ability to adoptively transfer EHP, despite appropriate changes of cytokine secretion patterns in BALB/c animals.

These data indicate that neither genetic background nor manipulation of cytokine environment at the time of initial antigenexposure *in vivo* substantially changes murine adoptive EHP. This suggests that attributes (including dose and route of administration) of the antigen determines the characteristics of the pulmonary response.

Methods

Animals

Male C3H/HeJCr and BALB/cAnCr mice (NCI, Frederick Cancer Research Facility, Frederick, Md) were housed in laminar flow hoods with HEPA filtered air in the Veterinary Medical Unit at the Albuquerque VA Medical Center which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Study design

Our design is to utilize our adoptive transfer model of experimental hypersensitivity pneumonitis in animals to attempt to alter the results of intratracheal (i.t.) administration of the agent, *Saccharopolyspora rectivirgula* (*S. rectivirgula*), which causes Farmer's Lung Disease in humans. The dependent variables are the extent of histological abnormalities and bronchoalveolar lavage characteristics. We compared the results of i.t. antigen exposure in mice that exhibit a Th2 bias (BALB/c) to those with a Th1 bias (C3H/HeJ). We also attempted to change the nature of the response by administering IL4 and anti-IFNγ.

Antigen

S. rectivirgula was obtained from V. Kurup, Medical School of Wisconsin, Milwaukee, Wis. and prepared as previously described (Schuyler *et al*. 1991, 1992, 1993, 1997).

Intratracheal (i.t.) inoculation

Mice were anaesthetized with a mixture of ketamine and xylazine. Lyophilized *S. rectivirgula* was suspended in sterile pyrogen free normal saline and injected into the trachea per os.

Cell preparation and culture

Lung associated lymph nodes (LALN) were removed from the right hilar and right mediastinal area and dispersed. Spleens were removed and splenic cells dispersed into medium by pressing spleen pieces between the frosted ends of two sterile microscope slides and filtered through a 70-mm cell strainer. Erythrocytes were lysed by exposure to 0.15 M NH_4Cl, the cells washed, and cultured in RPMI 1640 with penicillin, streptomycin, 2 mM glutamine, 5×10^5 M 2-mercaptoethanol and 10% in tissue culture dishes at 2×106 cells/ml in a 5% CO_2 humidified atmosphere for 72 h with a soluble extract of *S. rectivirgula* (30 μg/ml). Cultures were harvested by centrifugation, supernatants frozen at -70°C for cytokine assays, cells washed, viability determined using trypan blue dye exclusion and 5×10^6 LALN or 20×10^6 spleen cells injected intravenously (i.v.) in 0.3 ml volume into the tail veins of recipients.

In some experiments, mouse lungs were lavaged prior to LALN removal. The trachea was cannulated and 6×1 ml washes of normal saline were collected and centrifuged. Bronchoalveolar lavage (BAL) cells were

resuspended in RPMI-10, counted, cytocentrifuge preparations made, stained with Diff-Quick (Dade Diagnostics, AguAdA, P.R. USA), and evaluated for cell type.

Antibody isotypes

The amounts of serum specific anti-*S. rectivirgula* IgG1 and IgG2a and total IgG1 and IgG2a were measured by ELISA. In brief, a 96 well polycarbonate plate (NUNC, Naperville, IL) was coated with 10μg/ml (in PBS) soluble *S. rectivirgula*, or either goat antimouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL, USA). Skim milk 2.5% was used to block nonspecific binding sites and to dilute reagents and sera samples. After washing four times with PBS with Tween 20, sample and/or standard solution were added to each well and incubated at 37°C for 1 h. Horseradish peroxidase labelled goat antimouse IgG1 or IgG2a (Southern Biotechnology Associates) were added to each well, with appropriate washing and incubation steps before addition of an ABTS substrate. Plates were incubated for 30 min at room temperature and absorbance read at 405 nm with a reference wavelength of 550 nm. Unknown serum specific samples were expressed as optical density units (ODU) which is the optical density multiplied by the dilution factor. Unknown total IgG1 and IgG2a samples were compared with standard curves of IgG1 and IgG2a and expressed as ng/ml.

Cytokines

IFNγ and IL10 were measured using an ELISA (Schuyler *et al*. 1997). IL4 was measured using an IL4 responsive cell line (CT4.S) (Fei *et al*. 1995). IL5 was measured by ELISA using the Interleukin-5 Minikit (KM-IL5) from ENDOGEN (Cambridge, MA). The lower limit of the assays sensitivity are: IL4 : 1.25 pg/ml; IFNγ: 600 pg/ml; IL5: 30 pg/ml; IL10: 62.5 pg/ml.

IL4 and anti-IFNγ

IL4 was obtained from cultures of CHO cells transfected with the IL4 gene (kindly provided by Dr Joan Stein-Streilein, University of Miami School of Medicine) and purified using the Affi-Gel H2 Immunoaffinity kit (BioRad, Richmond, CA). Anti-IFNγ clones R4–6A2 and XMG1.2 were obtained from ATCC, grown in pristane induced ascitic fluid of nude mice and antibody purified by caprylic acid clarification, saturated ammonium sulphate precipitation and ion exchange chromatography. Control animals were treated with 1% BSA (the carrier for IL4) and rat isotype control.

Table 1. Schedule

Day	0	2	4	7	11	25	35
i.t.	+	+	+				
Sera	+				+	+	+
Sacrifice				+			+

i.t., Intratracheal injection of 7.2 mg/kg *S. rectivirgula*.

Histological studies

The trachea was cannulated and the lungs inflated with pH 6.9 buffered formalin under 20 cm water pressure for 48 h. After inflation, the lungs were sectioned (transverse for mediastinal lobe and sagittal for the other 4 lobes), embedded in a single paraffin block, and a 5 micron section cut and stained with haematoxylin and eosin. The slides were evaluated without knowledge of treatment. The area covered by an eyepiece grid (0.99 mm $\times$ 0.99 mm using $\times$100 magnification) was judged to be normal or abnormal. An abnormal field is one with increased number of cells in the interstitium or alveoli or both. An average of 300 fields were evaluated from each mouse (50% of the area under the cover slip).

Immunohistochemistry

Some animals lungs were prepared for frozen sections by inflation at 30 cm hydrostatic pressure via the trachea

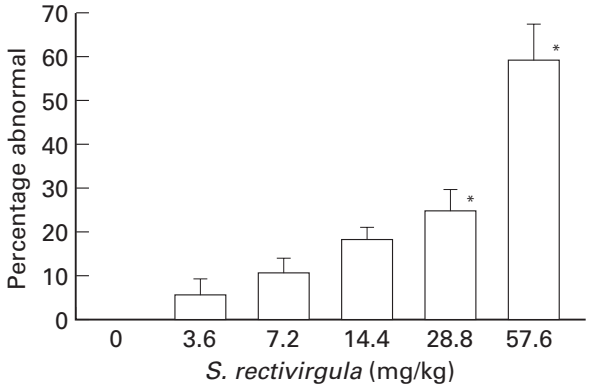

Figure 1. Extent of pulmonary histological abnormalities in animals receiving differing amounts of *S. rectivirgula* intratracheally. Animals were sacrificed 96 h after receiving a specified amount of *S. rectivirgula*. Percentage abnormal is percentage of 300 microscopic fields/animal that are abnormal. Six animals per group. Values are expressed as mean and standard error of the mean. *$P<0.05$ compared to 3.6 mg/kg *S. rectivirgula*. 0 mg/kg is different ($P<0.05$) from all other values. (Analysis of variance with Tukey's hsd procedure).

Table 2. Total serum immunoglobulin isotypes after immunization with *S. rectivirgula*

Mouse strain	Isotype	Day 0	Day 11	Day 25	Day 35
BALB/c	IgG_1	209.4 ± 54.5	312.6 ± 82.3	426.1 ± 87.9	413.8 ± 58.8
	IgG_{2a}	78.5 ± 28.2	101.3 ± 35.9	117.2 ± 39.1	163.1 ± 46.9
C3H/HeJ	IgG_1	51.6 ± 7.8	238.8 ± 60.9	194.1 ± 55.6	55.6 ± 18.3
	IgG_{2a}	50.0 ± 11.9	184.3 ± 40.0	168.6 ± 47.9	164.3 ± 25.0

Mean (ng/ml) ± s.e.m. $n = 5–7$. Days refer to time since the first of 3 i.t. injections of 7.2 mg/kg *S. rectivirgula*.

Total IgG_1 is higher ($P < 0.05$) in the BALB/c than the C3H/HeJ mice, but total IgG_{2a} is not different between the strains. Total IgG_1 increased after immunization in the BALB/c mice (Multiple ANOVA and Fisher's lsd method of post hoc multiple comparisons).

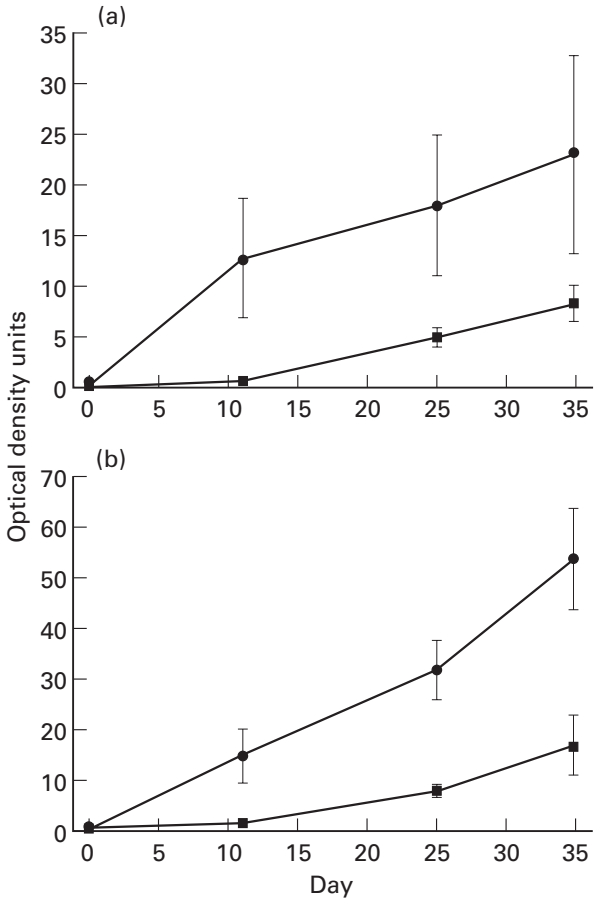

Figure 2. Anti-*S. rectivirgula* serum antibody in BALB/c (■) and C3H/HeJ (●) mice after 3 i.t. inoculations with *S. rectivirgula*. Results are expressed as Optical density units which are the optical density multiplied by the dilution factor. a, Antigen specific IgG_1. b, Antigen specific IgG_2. Mean, standard error of the mean. Two-way analysis of variance indicated that there was a significant ($P < 0.05$) effect of both the strain classification (i.e. BALB/c and C3H/HeJ), and the time from immunization.

with a 1:3 solution of O.C.T. (Tissue Tek, Miles, Inc., Elkhart, IN) and 20% sucrose in phosphate buffered saline. The lungs were quickly frozen in isopentane followed by liquid nitrogen, and stored at −80°C until sectioned at 8 μm on a cryostat.

To evaluate locations and relative proportions of CD4 and CD8 lymphocytes, serial frozen lung sections were placed on charged glass slides, fixed in 4°C acetone for 10 s, and immunostained as described in detail by Nikula *et al.* (1997). Briefly, endogenous peroxidase was blocked with H_2O_2 and serial slides from each mouse were reacted with rat antimouse IgG2a monoclonal antibodies (PharMinigen, Sorrento Valley, CA) to detect mouse CD4+ (CD4; L3T4) and CD8+ (CD8a; Ly-2) lymphocytes. Isotype-specific rat immunoglobulins (IgG2a; PharMinigen) were substituted for primary antibodies on serial immunoglobulin (negative) control slides. Biotinylated rabbit antirat (mouse adsorbed) IgG secondary antibody (Vector Laboratories, Burlingame, CA) followed by an avidin-biotin peroxidase system (ABC Elite Kit; Vector Laboratories) and 3,3(−diaminobenzidine were used to detect bound CD4 and CD8 antibodies. The slides, which were not counterstained, were evaluated using light microscopy.

Table 3. Culture supernatant cytokines

Mouse strain	Days	IL4	IFNγ
BALB/c	0	ND	ND
	7	1.5 ± 1.5	ND
	35	1.5 ± 1.5	ND
C3H/HeJ	0	3.2 ± 1.2	ND
	7	1.4 ± 0.8	1515 ± 95
	35	2.9 ± 1.2	ND

Antigen (30 μg/ml *S. rectivirgula*) stimulated spleen cell cultures (2×10^6/ml, 72 h). All values are ρg/ml. Days, Days after first of 3 i.t. injections of *S. rectivirgula*. 0, Cells from naïve animals. ND, None detected. Mean ± s.e.m. $n = 3–5$.

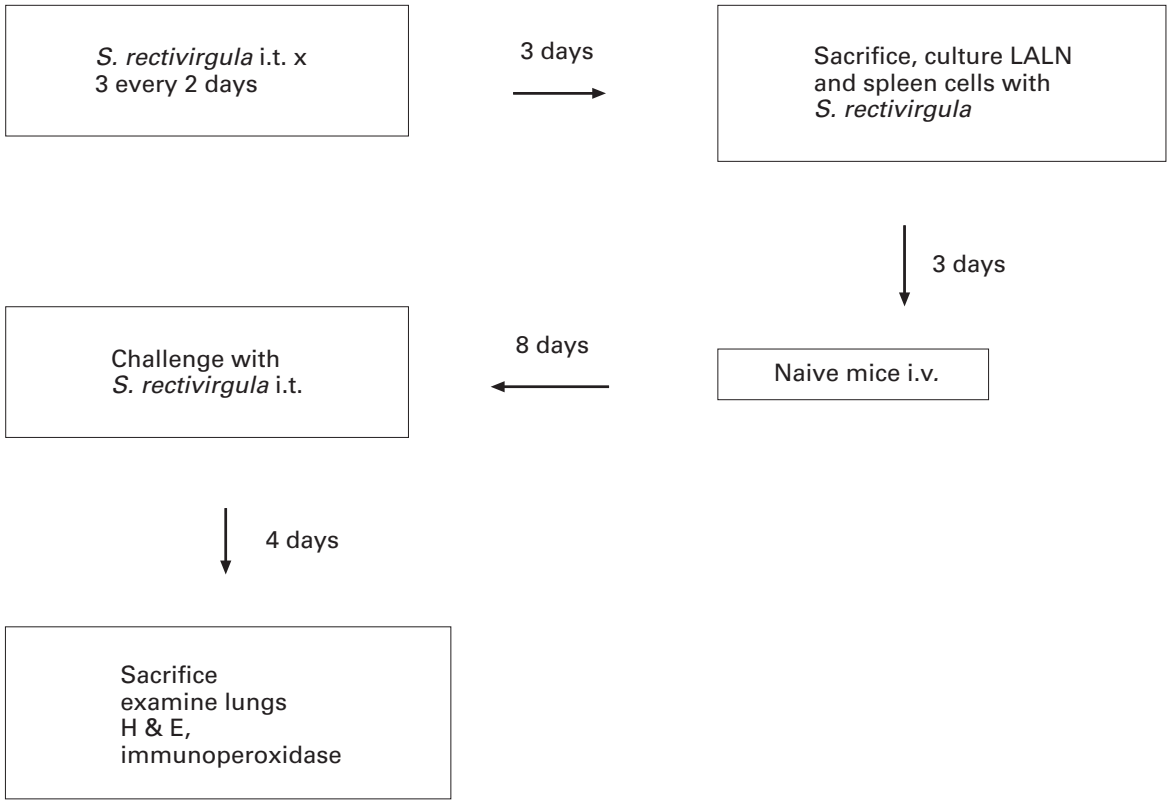

Figure 3. Experimental design of adoptive transfer model. i.t., intratracheal; i.v., intravenous; LALN, Lung associated lymph nodes; H & E, Hematoxylin and eosin.

Data analysis

Since the percent abnormal (p^) histological fields is obtained from binary outcomes, we first applied the transformation designed to equalize the variances of such(binomial like) responses: $y =$ arcsin (square root (p^) (Feinberg 1980). A concurrent control (RPMI) group was used with each experiment. Analysis of variance using Tukey's hsd procedure was used to compare groups. Multiple ANOVA and Fisher's lsd method of post

Table 4. Culture supernatant immunoglobulin (Total)

Mouse strain	Days	IgG_1 (ng/ml)	IgG_{2a} (ng/ml)
BALB/c	0	0.87 ± 0.023	0.60 ± 0.135
	7	6.92 ± 3.65	1.28 ± 0.42
	35	7.99 ± 1.56	5.57 ± 1.23
C3H/HeJ	0	1.01 ± 0.30	3.58 ± 1.05
	7	77.80 ± 32.69*	37.72 ± 8.21*
	35	3.60 ± 0.44	6.69 ± 0.75

Antigen (30 μg/ml *S. rectivirgula*) stimulated spleen cell cultures (2×10^6/ml, 72 h). Mean, s.e.m. Days, Days after first of 3 i.t. injections of *S. rectivirgula*. 0, Cells from naïve animals. *n*, 3–5. *$P<0.05$ compared to all other values (ANOVA with Tukey's hsd comparison).

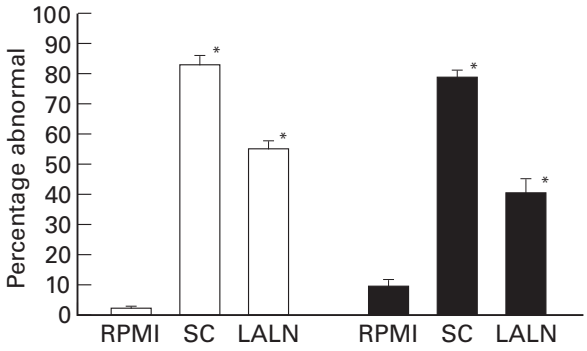

Figure 4. Extent of pulmonary histological abnormalities in BALB/c (□) and C3H/HeJ (■) recipients of 20×10^6 cultured spleen cells or 5×10^5 lung associated lymph node cells 4 days after intratracheal challenge with *S. rectivirgula*. Cells were transferred 8 days before intratracheal challenge. Percentage abnormal, percentage of 300 microscopic fields per animal that are abnormal; RPMI, animals treated with RPMI i.v.; LALN, Lung associated lymph nodes. Five to 12 animals per group. *$P<0.05$ compared to RPMI group. There are no differences among either the spleen cell or the LALN cell groups (Analysis of variance with Tukey's hsd procedure).

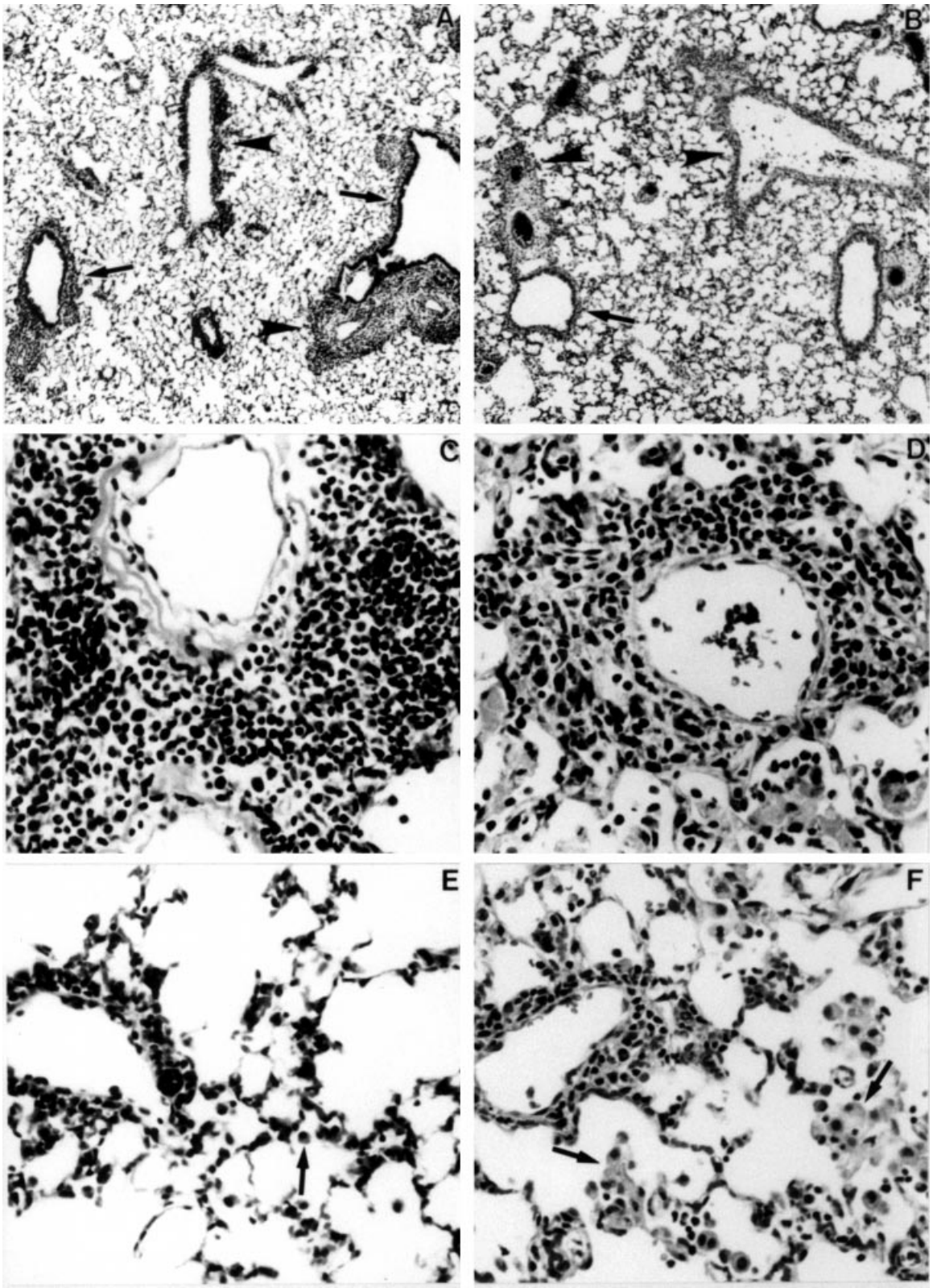
A
B
C
D
E
F

Table 5. Bronchoalveolar lavage characteristics C3H/HeJ and BALB/c

	None	IL4+ Anti-IFNγ	BSA + Rat IgG1
C3H/HeJ			
Total cell number ($\times 10^{-5}$)	7.75 ± 0.72	3.59 ± 0.54	10.38 ± 4.58
Macrophage number ($\times 10^{-5}$)	4.98 ± 0.52	2.45 ± 0.32	6.24 ± 2.67
Lymphocyte number ($\times 10^{-5}$)	1.03 ± 0.16	0.44 ± 0.09	0.98 ± 0.22
Neutrophil number ($\times 10^{-5}$)	1.65 ± 0.36	0.63 ± 0.18	2.90 ± 1.64
Eosinophil number ($\times 10^{-5}$)	0.09 ± 0.05	0.07 ± 0.03	0.26 ± 0.19
BALB/c			
Total cell number ($\times 10^{-5}$)	6.24 ± 2.21	1.74 ± 0.20	2.24 ± 0.43
Macrophage number ($\times 10^{-5}$)	2.84 ± 0.88	1.19 ± 0.07	1.31 ± 0.22
Lymphocyte number ($\times 10^{-5}$)	0.73 ± 0.27	0.15 ± 0.03	0.24 ± 0.05
Neutrophil number ($\times 10^{-5}$)	2.61 ± 1.08	0.38 ± 0.11	0.68 ± 0.19
Eosinophil number ($\times 10^{-5}$)	0.07 ± 0.05	0.02 ± 0.01	0.02 ± 0.01

All animals received one i.t. injection with *S. rectivirgula*. IL4, 5 × 1 μg IL-4 intraperitoneal (i.p.) injections (−1, 0, 1, 2, 3) days post i.t. *S. rectivirgula*). Anti-IFNγ, Anti-IFNγ (clone R4–6A2), 2 mg i.p. 2 days prior to i.t. *S. rectivirgula*). BSA, 1% bovine serum albumin (carrier for IL4). Rat IgG1, Rat isotype control. Mean ± s.e.m. 6–7 experiments per group. There are no differences between the groups (ANOVA with Tukey, s hsd comparison).

hoc multiple comparisons was used to compare the effect of cell source and time (Systat 1997).

Results

Determination of pulmonary histological response of BALB/c mice to i.t. S. rectivirgula

Animals were injected intratracheally once with varying amounts of *S. rectivirgula* and sacrificed 4 days thereafter. The extent of pulmonary inflammation was dependent on the amount of injected *S. rectivirgula* (Figure 1). The slope of the curve and the y-intercept of the relationship between amount of injected *S. rectivirgula* in BALB/c mice was similar to that of C57Bl/6, SJL/J and C3H/HeJ mice (Schuyler *et al*. 1991).

Comparison of response of BALB/c to C3H/HeJ to 3 i.t. injections of S. rectivirgula

Serum antibody isotypes. Animals were treated with 3 i.t. injections of 7.2 mg/kg *S. rectivirgula* every other day. Specific anti-*S. rectivirgula* IgG1 and IgG2a antibody and serum total IgG1 and IgG2a were measured on day 0, 11, 25 and 35 after the first i.t. exposure to *S. rectivirgula* (Table 1).

C3H/HeJ mice exhibited more serum IgG1 and IgG2a anti-*S. rectivirgula* antibody than BALB/c mice (Figure 2) (Multiple ANOVA and Fisher's lsd method of post hoc multiple comparisons). Two-way analysis of variance indicated that there was a significant ($P < 0.05$) effect of both the strain classification (i.e. BALB/c and C3H/HeJ), and the time from immunization. Total IgG1 was higher inthe BALB/c than the C3H/HeJ mice, but total IgG2a was not different between the strains (Table 2). In addition, total IgG1 increased after immunization (Multiple ANOVA and Fisher's lsd method of post hoc multiple comparisons).

Cytokine and immunoglobulin profile of in vitro *cultures*. We analysed cytokine profile and immunoglobulin secreted into spleen cell cultures. Spleens were removed and cultured with 30 mg/ml *S. rectivirgula* for 72 h 7 or 35 days after the first i.t. *S. rectivirgula* inoculation.

We detected very small amounts of IL4 in cultures from both BALB/c and C3H/HeJ mice, but substantial amounts of IFNγ in supernatants of cultures from C3H/HeJ animals sacrificed 7 days after the first of 3 i.t. injections (Table 3).

After 72 h of culture, we detected substantial amounts of total IgG1 and IgG2a in culture supernatants from both C3H/HeJ and BALB/c immunized mice with more IgG1 and IgG2a in supernatants from cultures from C3H/HeJ

Figure 5. Histopathological characteristics of adoptively transferred EHP. A, Diffuse interstitial pneumonia in a BALB/c mouse. Note the cellular cuffs around bronchioles (arrows) and veins (arrowheads (× 60). B, Interstitial pneumonia in a C3H/HeJ mouse. Cuffs around bronchioles (arrows) and veins (arrowheads) are less prominent compared to the BALB/c mouse. Close inspection shows more cells in alveoli in the C3H/HeJ mouse (× 60). C, Predominantly lymphocytic perivascular cuff in a BALB/c mouse (× 360). D, Pleocellular perivascular cuff in a C3H/HeJ mouse. Numerous macrophages and neutrophils are present in the lumens of adjacent alveoli (× 360). E, Small perivascular lymphocytic cuff and alveolar septal infiltrates in a BALB/c mouse. Few cells are present in alveolar lumens; those present are small macrophages (arrow) (× 300). F, Small perivascular pleocellular cuff and intra-alveolar cellular infiltrate. Alveolar macrophages have abundant cytoplasm (arrows) (× 300).

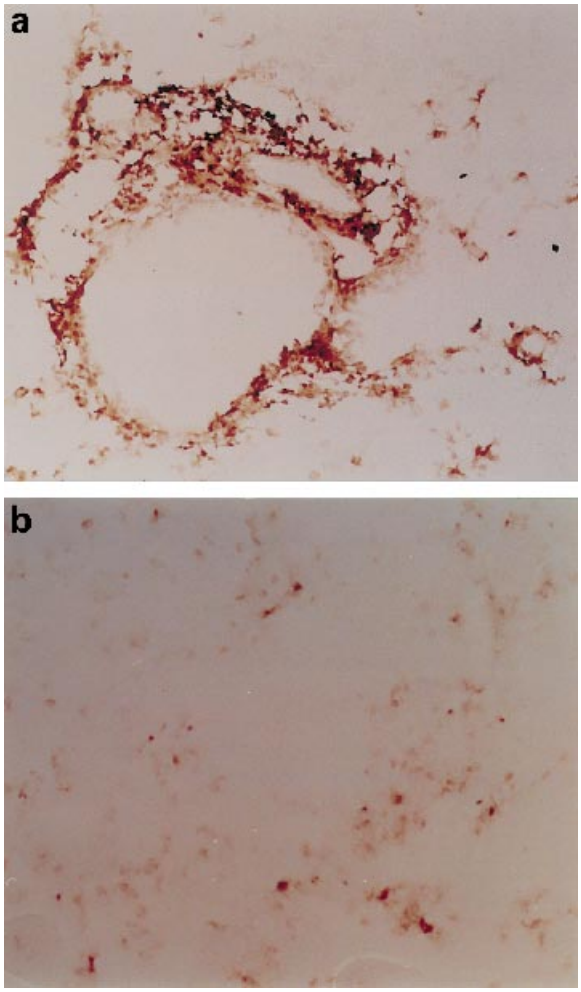

Figure 6. Immunohistochemistry. A, Perivascular cuff in a C3H/HeJ mouse immunoreacted with anti-CD4 primary antibody. CD4 cells in the cuff and adjacent alveolar septa are stained grey to black in this photograph (×180). B, Serial section immunoreacted with anti-CD8 primary antibody. Only a few cells are stained. Example shown is from a C3H/HeJ mouse; immunophenotyping results were the same in both strains (×180).

cells obtained 7 days after the first of 3 i.t. exposures to *S. rectivirgula* (Table 4).

Adoptive transfer of EHP

Donor animals were sensitized with 3 i.t. inoculations of 7.2 mg/kg *S. rectivirgula* (Day 0, 2 and 4) and sacrificed 3 days after the last challenge (Day 7) (Figure 3). LALN and spleen cells were cultured, the cells were harvested and transferred to recipient animals. Eight days thereafter, mice were challenged i.t. with 7.2 mg/kg *S. rectivirgula* and sacrificed by exsanguination four days later for pulmonary histological examination.

Cell culture. The yield from culture and the proportion of cells with high cytoplasmic/nuclear ratio (i.e. blast cells) was similar in the two mouse strains (data not shown).

Histology. The extent of histological abnormalities was similar in these 2 strains of mice (Figure 4).

Although the severity of pneumonia was similar in BALB/c and C3H/HeJ mice four days after administration of *S. rectivirgula*, there were differences in the histological characteristics of the response. BALB/c mice exhibited diffuse interstitial pneumonia with marked perivascular and peribronchiolar cuffing (Figure 5A). The cuffs were densely cellular, and the majority of the cells within the cuffs were lymphocytes (Figure 5C). Mitotic lymphocytes were frequent. Lesser numbers of eosinophils and monocytes were present in the cuffs. There were mild septal alveolar infiltrates of lymphocytes, eosinophils, macrophages, and neutrophils. Few cells were present in the alveolar lumens of BALB/c mice (Figure 5E). When present, lumenal cells were primarily small macrophages.

C3H/HeJ mice exhibited multifocal to diffuse interstitial pneumonia with relatively more intralumenal cells than BALB/c mice (Figure 5B). The perivascular and peribronchiolar cuffs were more pleocellular in C3H/HeJ mice (Figure 5D). Unlike BALB/c mice, which had cuffs around all sizes of veins and venules distributed throughout the lungs, the cuffs in C3H/HeJ mice were less evenly distributed and tended to occur in foci with intralumenal cellular exudates (Figures 5D, 5F). The cells in the cuffs and septal infiltrates were monocytes, eosinophils, lymphocytes, and neutrophils. Alveolar lumenal exudates consisted of macrophages and neutrophils. The macrophages had abundant, frequently vacuolated cytoplasm (Figures 5D, 5F).

Immunohistochemistry. The lymphocytes in perivascular and peribronchiolar cuffs and in septal infiltrates were CD4+ T lymphocytes in both strains of mice (Figures 6A, 6B). CD8+ lymphocytes were infrequent and scattered throughout the lungs (Figures 6A, 6B).

Treatment with IL4 and anti-IFNγ

We attempted to alter the nature of the immune response of both Balb/c and C3H/HeJ mouse strains to *S. rectivirgula* by treating animals with IL4 and anti-IFNγ during the sensitization period (Figure 7). We determined in preliminary studies that one i.t. exposure to *S. rectivirgula* is sufficient to produce cells that can adoptively transfer EHP after culture in both mouse strains.

Three different treatment protocols were used to determine the effects of IL-4 with or without added anti-IFNγ on *S. rectivirgula* sensitized mice, using doses of IL4 and

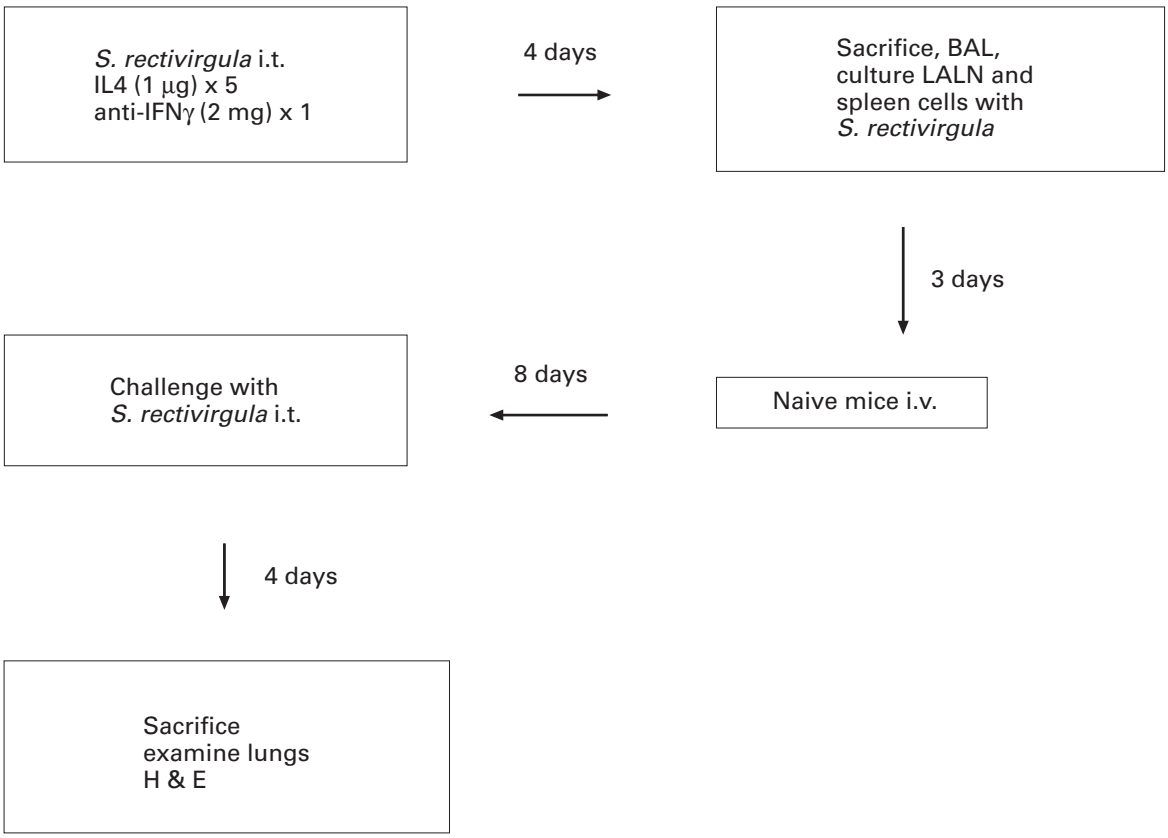

Figure 7. Experimental design of modulation of adoptive transfer model. i.t., intratracheal; i.v., intravenous; LALN, Lung associated lymph nodes; BAL, Bronchoalveolar lavage; H & E, Hematoxylin and eosin.

anti-IFNγ comparable to those which changed the immune response to *Leishmania* (Scott 1991; Chatelain *et al*. 1992), *Schistosomiasis* (Smythies *et al*. 1992), cerebral malaria (Grau *et al*. 1989), cardiac transplantation (Piccotti *et al*. 1998) and models of autoimmune diseases (Racke *et al*. 1994).

First, BALB/c mice which had received a single i.t. *S. rectivirgula*, followed by 7 × 1 mg IL-4 intraperitoneal injections (0, 12, 24, 36, 48, 60, 77 h post i.t.), were sacrificed 4 days post i.t. Control animals were treated with 1% BSA (the carrier for IL4). Spleen and BAL cells were collected, and spleen cells were cultured with 30 μg/ml *S. rectivirgula* for transfer into recipient mice which were subsequently challenged 8 days later with a single i.t. *S. rectivirgula*, and sacrificed for histological observations 4 days post challenge i.t. We found no effect of treatment of donor animals with IL4 or BSA on the ability of cells to adoptively transfer

Table 6. Culture supernatant cytokines

Cell source	Treatment	IL4	IL5	IL10
Spleen	Nil	5.4 ± 0.6	26 ± 7	70 ± 7
	IL4 + anti-IFNγ	29.9 ± 8.0	135 ± 30	160 ± 29
	BSA + rat IgG	8.7 ± 3.7	53 ± 14	101 ± 18
LALN	Nil	14.2 ± 6.3	30 ± 10	153 ± 73
	IL4 + anti-IFNγ	46.7 ± 19.9*	275 ± 170*	456 ± 207*
	BSA + rat IgG	8.7 ± 2.2	23 ± 5	148 ± 24

BALB/c mice. Antigen (30 μg/ml *S. rectivirgula*) stimulated cell cultures (2×10^6/ml, 72 h). Mean, s.e.m. All cytokine concentrations pg/ml. LALN, Lung associated lymph nodes. IL4 + anti-IFNγ, 5 × 1 μg IL-4 (−1, 0, 1, 2, 3 days post i.t. *S. rectivirgula*); plus 2 mg intraperitoneal anti-IFNγ (clone R4–6A2), 2 days prior to i.t. *S. rectivirgula*. BSA + rat IgG, 5 injections of 1% BSA using the same schedule as IL4 plus 2 mg of rat IgG using the same schedule as anti-IFNγ. Nil, No treatment. *n*, 5–12. *$P < 0.05$ compared to both Nil; and IL4 + anti-IFNγ treatments (ANOVA with Tukey's hsd comparison).

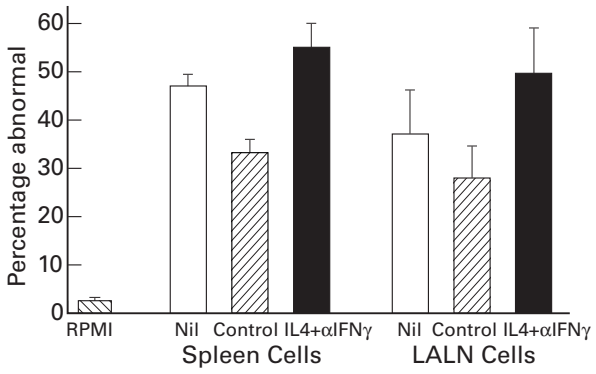

Figure 8. Extent of pulmonary histological abnormalities in BALB/c recipients of 20×10^6 cultured spleen cells or 5×10^5 lung associated lymph node cells 4 days after intratracheal challenge with *S. rectivirgula*. Cells were transferred 8 days before intratracheal challenge. Percentage abnormal, percentage of 300 microscopic fields per animal that are abnormal; RPMI, Animals treated with RPMI i.v.; Control, Donor animals treated with BSA (carrier for IL4) and isotype control antibody; Nil, Donor animals not treated; IL4+ αIFNγ, Donor animals treated with IL4 and anti-IFNγ antibody; LALN, Lung associated lymph nodes. Five to 12 animals per group. The RPMI group is different ($P<0.05$) from all spleen cell groups and all LALN groups. There are no differences among either the spleen cell or the LALN cell groups (Analysis of variance with Tukey's hsd procedure).

EHP (data not shown), or in bronchoalveolar cell characteristics (data not shown).

Next we added 2 mg intraperitoneal injections of anti-IFN g (clone R4–6A2) or normal rat IgG (Jackson ImmunoResearch Labs, West Grove, PA), 2 days prior to i.t. *S. rectivirgula* combined with 5×1 mg IL-4 or 1% BSA (-1, 0, 1, 2, 3 days post i.t.). We also examined the ability of cultured LALN cells to adoptively transfer EHP and the cytokine content of supernatants of cultures of both spleen and LALN cells. There were no differences of the numbers of cells in the LALN and spleens harvested from both BALB/c and C3H/HeJ animals treated with IL4 and anti-IFNγ, compared to those treated with BSA and rat isotype control (data not shown). Treatment of BALB/c or C3H/HeJ donor animals with IL4 plus anti-IFNγ had no effect on the ability of either spleen or LALN cells to transfer EHP (Figure 8 and data not shown). There were also no differences of bronchoalveolar lavage parameters (Table 5). However treatment did change the cytokine profile of culture supernatants of BALB/c LALN cell cultures. The amounts of IL4, IL5 and IL10 (Table 6), but not IFNγ (data not shown), were increased in supernatants from cultures of LALN cells from animals treated with IL4 plus anti-IFNγ, compared to untreated animals, or animals treated with control antibody and BSA. Similar treatment of BALB/c mice with IL4 and another anti-IFNγ monoclonal antibody (XMG1.2, 2 mg each at 2 days before, at the time, and 2 days post i.t. *S. rectivirgula*) produced comparable results in that ability to adoptively transfer was not altered (data not shown).

Discussion

Hypersensitivity pneumonitis is characterized by clinical illness occurring in only a minority of exposed subjects despite evidence of sensitization (i.e. serum antibody and bronchoalveolar lavage lymphocytosis) in most exposed subjects. The reasons for this phenomenon are unknown, but could include characteristics of the host (including the nature of the immune response), or the agent that causes HP. Differences of genetic backgrounds as reflected by HLA haplotypes or TCR usage have not demonstrated differences between exposed but not ill and exposed and ill subjects (Christensen *et al*. 1975; Rittner *et al*. 1983; Selman *et al*. 1987; Terho *et al*. 1987; Richeldi *et al*. 1993). However, there are differences in isotypes of antibody directed against agents that cause HP, that suggest that more subtle differences of immune response might determine the result of antigen exposure. Pigeon breeders who remain asymptomatic have a higher level of serum IgG4 antibody directed against pigeon antigens than those who develop HP (Kitt *et al*. 1986). IgG4 is characteristic of a Th2 response in humans (Ishizaka *et al*. 1990; Spiegelberg *et al*. 1991; Schmitt *et al*. 1994).

Our model of adoptive transfer of EHP allows examination of the role of genetic factors and cytokine environment at the time of initial antigen exposure by separating sensitization, development of effector cells and the results of interaction of effector cells and antigen. Cells from animals sensitized to *S. rectivirgula*, previously known as *Micropolyspora faeni*, the organism responsible for Farmer's Lung Disease in humans (Schuyler 1997), are cultured in the presence of *S. rectivirgula* and injected into recipients. The recipient animals exhibit exaggerated pulmonary histological response to intratracheal (i.t.) challenge with *S. rectivirgula*. This phenomenon is dependent on sensitization of the donors, culture of the cells with antigen, concentration of antigen and the number of transferred cells (Schuyler *et al*. 1991). This model (administration of cultured cells from sensitized animals before i.t. challenge with *S. rectivirgula*), is designated as adoptive EHP (Figure 3).

Current concepts of immunological reactivity derive from seminal observations by Mosmann and Coffman who reported that murine CD4+ cell clones can be driven *in vitro* to one of several patterns of cytokine secretion (Mosmann *et al*. 1986). Th1 cells preferentially secrete

IFNγ and tumour necrosis factor-β (TNFβ); activate macrophages; are responsible for cell-mediated immunity reactions and CTL (cytotoxic lymphocytes); and provide help for IgG2a production. Th2 CD4+ cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13; provide help for immunoglobulin (particularly IgE and IgG1) secretion; enhance eosinophil production, survival, and activity; and promote mast cell proliferation and maturation. Development of either antigen specific Th1 or Th2 response after *in vivo* exposure in mice is dependent on many factors, including attributes of the antigen; site of antigen delivery; adjuvant used; age, sex and genetic background of the mouse; nature of the antigen-presenting cell encountered; and involvement of different costimulatory molecules (Gause *et al*. 1997). Cytokines secreted by one CD4+ subset inhibit the development of the reciprocal subset leading to a predominance of one of the subsets and polarization of the antigen specific immune response (Finkelman 1995; Ria *et al*. 1998). IL-4 is particularly important in inducing differentiation of Th2 cells, and IL-12 and IFNγ in inducing differentiation of Th1 cells, from Thp (Th precursor) cells. Irreversible commitment to a Th1 or Th2 phenotype is probably mediated by rapid loss of the ability of IL-4, or IL-12 and IFNγ, to initiate intracellular signalling in differentiated Th1 or Th2 cells, respectively (Guler *et al*. 1997; Nakamura *et al*. 1997; Szabo *et al*. 1997). This lack of responsiveness of differentiated Th2 or Th1 cells is caused by either changes in IL4 or IL12 receptors or in cytoplasmic and/or nuclear signal transduction factors, such as phosphorylation of Janus and STAT kinases (Bacon *et al*. 1995; Jacobson *et al*. 1995; Pernis *et al*. 1995; Guler *et al*. 1997; Szabo *et al*. 1997).

Recently, it has become evident that the above formulation (Th1 or Th2 cells) may be overly simplistic in that resting memory differentiated Th cells can produce cytokines characteristic of both Th1 and Th2 cells, and that administration of IL12 can increase the antigen specific production of both IFNγ and IL4 by CD4+ cells (Bliss *et al*. 1996). However, the concept that the cytokine milieu (derived from many possible sources, such as macrophages, dendritic cells, CD4+ cells, CD8+ cells, NK cells, mast cells, eosinophils, $\gamma\delta$ T cells) at the time of antigen specific CD4+ cell differentiation, determines the later pattern of cytokine secretion and function of the differentiated cells is valid.

IL12 is necessary for expression of HP in murine models of HP which use repetitive intrapulmonary administration of *S. rectivirgula* (Gudmundsson *et al*. 1998). Gudmundsson found that mouse strains differ in susceptibility to HP with C57Bl/6 more sensitive than DBA/2 mice (Gudmundsson *et al*. 1998). The increased sensitivity of the C57Bl/6 mice correlated with increased expression of IL12. The decreased susceptibility of the DBA/2 mice could be ablated by administration of IL12. These data support the concept that expression of EHP correlates with expression of IL12, the prototypic Th1 cytokine which drives differentiation of the immune response to a Th1 type of response. Our recent demonstration that Th1, but not Th2 cell lines, adoptively transfer EHP (Schuyler *et al*. 1997) also supports the concept that EHP is a Th1 disease. This is consistent with several animal models of autoimmune disease, such as autoimmune allergic encephalomyelitis, collagen induced arthritis and experimental allergic uveitis (Liblau *et al*. 1995).

Despite the strong Th2 bias of BALB/c mice, we did not find quantitative differences between BALB/c and C3H/HeJ mice in the pulmonary response to i.t. exposure to *S. rectivirgula*, or in the ability of cultured sensitized cells to adoptively transfer EHP (Figures 1 and 4). This is consistent with experimental allergic encephalomyelitis in which there was no difference between the response of BALB/c and C57Bl/6 mice to administration of a large amount of a crude antigen mixture (guinea pig spinal cord) (Falcone *et al*. 1998), but there were differences after administration of a component of spinal cord, proteolipid apoprotein (Tuohy *et al*. 1988). Since we administered a crude antigen mix, we might have obscured differences that would be evident if we used a single component.

In addition, Shibuya *et al*. (1998) recently demonstrated that IL1α and TNFα promote the differentiation of Th1 cells in BALB/c mice. *S. rectivirgula* causes IL1α and TNFα secretion from alveolar macrophages (Denis *et al*. 1991) and may therefore induce Th1 cells in BALB/c mice.

However, there were qualitative differences between the mouse strains in that the cellular infiltrate tended to include more intralumenal cells in the C3H/HeJ compared to the BALB/c animals. Both strains demonstrated interstitial infiltration with cells, but the cell mixture was more pleomorphic in the C3H/HeJ animals.

The amount of serum total IgG1 was higher in the BALB/c than the C3H/HeJ mice (Table 2) and spleen cells produced less IFNγ from BALB/c than from C3H/HeJ animals (Table 3), consistent with the Th2 bias of BALB/c animals. However, the amount of both IgG1 and IgG2a anti-*S. rectivirgula* serum antibody was higher in C3H/HeJ than BALB/c mice (Figure 2). This corresponded with *in vitro* secretion of these antibodies from spleen cells obtained 3 days after the last of 3 i.t. exposures to *S. rectivirgula* (Table 4). The larger amount of serum IgG2a specific antibody in C3H/HeJ (compared to BALB/c) animals is consistent with their Th1 bias. However, a similar increase of serum IgG1 specific antibody in

C3H/HeJ animals is somewhat surprising in view of the general conception that IgG1 is a Th2 cytokine. Results of administration of CpG oligodexynucleotides to BALB/c mice indicate that the amount of specific IgG1 antibody may not invariably reflect Th2 differentiation (Chu *et al*. 1997).

Treatment with IL4 and anti-IFNγ did not affect BAL cell characteristics (Table 5) or the ability of cultured cells to adoptively transfer EHP (Figure 8), but did change the cytokine profile (increased IL4, IL5 and IL10) in culture supernatants of LALN cells from treated compared to untreated BALB/c animals (Table 6). This indicates that our treatment did have an effect on the nature of the immune response to *S. rectivirgula*.

Therefore, unlike our ability to change the competence of cultured cells to adoptively transfer EHP by *in vitro* manipulation of the cytokine environment (Schuyler *et al*. 1997), we could not change these cells by *in vivo* manipulation of the cytokine environment present at the time of initial presentation of antigen. Our results are somewhat different from those obtained by Ghadirian & Denis (1992) who demonstrated a decrease in lung inflammatory response in mice administered *S. rectivirgula* treated with 1 μg IL4 weekly. However there are many differences between the experimental protocols, the most important of which is the mouse strain (C57Bl/6), and the use of multiple (9) injections of *S. rectivirgula* by Ghadirian & Denis (1992), compared to one injection in our protocol. We used a similar amount of IL4 for each IL4 treatment, but administered more treatments (5 compared to 3) over a shorter duration (4 days compared to 21 days). The lack of effect of treatment with anti-IFN γ on BAL cellularity is consistent with previous results using anti-IFNγ (Denis & Ghadirian 1992) and IFNγ knockout mice (Gudmundsson & Hunninghake 1997), and suggests that IFNγ is not necessary for *S. rectivirgula* induced lung inflammation as reflected by BAL cells.

Our results may be due to the adjuvant nature of *S. rectivirgula*, a thermophilic actinomycetes which is related to mycobacteria. *Saccharopolyspora rectivirgula* is a potent adjuvant (Bice *et al*. 1974) and mitogen (Smith *et al*. 1978), promotes the secretion of inflammatory cytokines from macrophages (Denis *et al*. 1991), and thus tends to produce a Th1 response (Shibuya *et al*. 1998). Future experiments will explore the ability of *S. rectivirgula* to induce the secretion of cytokines that cause differentiation of Th1 cells.

In summary, we found that differences in the nature of the murine immune response to intratracheally administered *S. rectivirgula*, either due to genetic factors or to manipulation of the cytokine environment at the time of initial antigen exposure, did not affect the extent of pulmonary histological changes caused by exposure to this material. In addition, genetic differences and cytokine manipulation *in vivo* did not affect the ability of cultured cells to adoptively transfer EHP. This implies that attributes of *S. rectivirgula*, rather than genetic or immunoregulatory differences in the host, determine the outcome of exposure to this agent. Based on these data, manipulation of cytokine environment *in vivo* would not be expected to change the response of humans to exposure to *S. rectivirgula*.

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