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Viral Infection Enhances the Response to *Saccharopolyspora rectivirgula* in Mice Prechallenged with this Farmer's Lung Antigen

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Abstract. Sendai viral infection enhances mice lung response to *Saccharopolyspora rectivirgula* (SR). The mechanisms of this viral enhancement remain unclear. The present study was done to verify if the viral infection was required and if the presence of the viral infection needed to be given simultaneously with the SR antigen for the enhanced response to occur. In a first experiment groups of C57BL/6 mice were instilled concomitantly with SR and live or inactivated Sendai virus. In a second experiment the viral infection in the appropriate group preceded the SR challenges by 4 weeks. As reported previously SR induced a cellular inflammatory response. This effect of SR was enhanced by the viral infection but not by inactivated virus particles. Total lavage cells 3 weeks after the virus inoculation in the appropriate groups were: saline = $69 \pm 15 \times 10^3$; SR = $678 \pm 104 \times 10^3$; Sendai = $277 \pm 61 \times 10^3$; inactivated Sendai = $73 \pm 23 \times 10^3$; SR + Sendai = $1232 \pm 232 \times 10^3$; SR + inactivated Sendai = $515 \pm 54 \times 10^3$. In the second experiment, where the infection preceded the SR challenge, no enhancement was observed. We conclude that Sendai virus enhances mice lung response to SR by an infectious process when both SR and the virus are present simultaneously.

Key words: Sendai virus—Hypersensitivity pneumonitis—Immune response

Introduction

Farmer's lung (FL) is one of the most common forms of hypersensitivity pneumonitis (HP). The agents responsible for this allergic disease are bacteria and fungi found most abundantly in moldy hay [19]. Although many exposed farmers have precipitating antibodies to these antigens, most of them do not develop overt disease [8, 14, 15].

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Despite this, half of the farmers with positive precipitins have a subclinical lymphocytic alveolitis [7]. Why most farmers remain asymptomatic whereas only a few go on to develop the disease is unknown. Studies looking for genetic markers to suggest a genetic predisposition to HP have mostly been negative [12, 16, 22].

Although HP results from an allergic reaction to specific organic or inorganic substances, its pathogenesis may involve the participation of more than a simple antigen. A trigger substance or event could be required to induce the hypersensitization, and once it is acquired only the presence of a specific allergen is needed to induce the disease. Evidence supporting the need of more than exposure to one antigen in the origin of HP is as follows. (1) Most animal models of HP require the addition of an adjuvant [20, 24]. (2) Patients with FL have a more intense reaction to total hay extract than to *Saccharopolyspora rectivirgula* (SR) alone [11, 25]. (3) Animals exposed to either glucans or lipopolysaccharide (LPS) develop a nonspecific inflammation, whereas those exposed to both substances have a HP-like granulomatous reaction [13].

Another important finding supporting the need for a sensitizing factor is our previous observation that a Sendai virus infection rendered mice hypersensitive to subsequent challenges with SR, the bacteria most frequently responsible for farmer's lung in Québec [6, 8, 9]. Animals infected with live Sendai virus developed a transient lung inflammatory response. This response peaked at about 9 days and disappeared within 3 weeks [3]. When mice presensitized by repeated SR instillations for 3 weeks were instilled with Sendai, they became hyperresponsive to SR challenges for at least 6 months after the Sendai infection; this was long after all signs of Sendai-induced inflammation had disappeared in mice instilled with the virus only. Moreover, Sendai + SR-treated animals developed lung lesions that more closely resembled FL than those treated with SR alone [9].

The mechanisms by which a Sendai infection induces this permanent sensitization are unknown. If Sendai virus acted only as an adjuvant, large quantities of inactivated viral particles should produce an enhancing effect similar to that of a viral infection.

Although no residual viral antigens were detectable in the lungs by standard techniques after a Sendai virus infection [2], it is possible that the virus infection induced hypersensitization by modifying the immune systems of the mice. If so, any infected animal would develop a hyperresponse to SR whether the virus was given concomitantly or before the antigenic preparation.

This study was done to verify whether the viral infection is required to induce hypersensitization to SR, and whether the viral infection has to be induced simultaneously with repeated SR challenges. Experiments were designed in which mice were given either inactivated viral particles or were infected with SR. Results suggest that a virus infection concomitant with SR exposure is required to confer hypersensitization to SR. Therefore, Sendai virus does not act simply as an additional antigen or as an adjuvant in its sensitization effect, and viral infection-induced modifications in the lungs are implicated in the hypersensitization to SR.

Materials and Methods

Animals

Pathogen-free C57BL/6 female mice were obtained from Charles River (Saint Constant, Québec). All animals were housed in filtertop cages. Sendai virus-infected animals were kept in a laminar flow hood in

a separate room isolated from the noninfected mice. Animals inoculated with inactivated Sendai virus were also isolated from other mice colonies.

Antigen

Lyophilized antigen was produced from a live culture of *S. rectivirgula,* as described previously [21], reconstituted with pyrogen-free saline at a concentration of 5 mg/ml, aliquoted, and stored at −70°C. A sample was tested in a limulus amebocyte lysate assay (Sigma Chemical Co., St. Louis MO) and was shown to be endotoxin free.

Virus

Parainfluenza 1 (Sendai) virus was obtained from the American Type Culture Collection, Rockville, MD, through the Institute Armand Frappier (Laval, Québec). The virus was propagated in the allantoic fluid of 10-day-old embryonating chicken eggs [5]. Stock virus titer was 4,096 HAU/50 μl. A 1:100 dilution of this virus suspension instilled intranasally in mice induces a moderate pneumonitis from which the animals recover readily [3]. Virus inactivation was done as described previously [18]. Briefly, the viral suspension was treated with 0.05% propiolactone (Sigma) overnight at 4°C and then for 1 h at 37°C followed by 10 min of UV irradiation 20 cm from a 30-watt germicidal lamp. Inactivation was confirmed by immunofluorescence. LLCMK2 cells were grown to near confluence in shell vials with coverslips, in Eagle's minimal essential medium with Earle's salts containing 10% fetal bovine serum and antibiotics. Prior to virus inoculation, the cell monolayer was washed three times with phosphate-buffered saline and fresh minimal essential medium containing bovine albumin (0.1%), D-glucose (0.2%), trypsin (2 units/ml), and antibiotics. Viral specimens (liver or inactivated Sendai virus) were inoculated (100 μl/vial), followed by a 1-h centrifugation at 1,500 ×*g*. Vials were then incubated in a CO_2 incubator at 36°C and observed daily for the presence of a cytopathic effect. Identification of positive specimens was confirmed by an indirect immunofluorescence assay using a monoclonal antibody for parainfluenza 1 (Baxter). Fifty μl of undiluted inactivated virus was used for intranasal instillations in appropriate animals.

Study Design

Inactivated Virus Experiment. Animals were lightly anesthetized with isoflurane, and 50 μl of the appropriate solution was deposited on the tip of the nose. The deposited solution was inhaled involuntarily by the animals. Six groups of mice were used. Group 1 received saline 3 days/week throughout the study period. Group 2 was given saline 3 days/week until the day of sacrifice and a single intranasal instillation of 40 HAU of Sendai virus 3 weeks after the beginning of the saline instillations. Group 3 was treated similarly to group 2 except that the animals were inoculated with concentrated inactivated Sendai virus. Experimental HP was induced in groups 4, 5, and 6 by giving 250 μg of SR in 50 μl for 3 successive days/week for 6 or 15 weeks. After 3 weeks of SR inoculations, mice from group 5 were infected with a single instillation of live Sendai virus, whereas group 6 animals were inoculated with inactivated virus. Inactivated Sendai virus particles were given 4 days later than the liver virus inoculants. This was done to coincide with the peak viral replication that occurs 4–5 days after the inoculation. Animals from all groups were sacrificed at either 3 and 12 weeks following the viral inoculation; that is, after 6 or 15 weeks of SR in the appropriate groups. The studied groups can be summarized as follows: group 1 = saline; group 2 = Sendai infection; group 3 = inactivated Sendai; group 4 = SR; group 5 = SR + Sendai infection; group 6 = SR + inactivated Sendai.

Prechallenge Viral Infection. Four groups of mice were studied. On day 1 two groups were instilled with a single inoculation of 40 HAU of Sendai virus. On day 28 one of these groups was started on three times weekly instillations of SR; the other virus-infected group received saline alone until sacrifice. One of the two noninfected groups of mice served as control and received saline instillations only; the other noninfected group received an instillation of SR similar to that received by the infected animals. All animals were anesthetized similarly with isoflurane for each saline or SR instillation. Animals from each group were

sacrificed by cervical dislocation 1, 2, 3, or 4 weeks after the beginning of the SR instillations in the appropriate groups. All sacrifices were done 4 days after the final intranasal instillation of saline or SR. Groups for this protocol were: group 1 = saline; group 2 = Sendai; group 3 = SR; group 4 = Sendai + SR.

Study Variables

The response to different challenges was evaluated by analysis of the total cell count and differential cell count of bronchoalveolar lavage fluid (BALF).

Cellular BALF Analysis

For cell examination, animals from the different groups studied were killed 3 days after the last instillation of either saline or SR. The trachea was cannulated with a 20-gauge plastic catheter and the lungs infused with three aliquots of 1 ml of sterile saline. BALF recovered was centrifuged and the cells resuspended in Hanks' balanced salt solution. Cell viability was assessed by trypan blue exclusion and the number of cells counted with a hemocytometer. Differential cell counts from each mouse were performed using Diff-quick and esterase stainings.

Statistical Analysis

Data were expressed as mean values ± S.E. for graphical representation. For comparisons between group means, a one-way analysis of variance (ANOVA) was performed. Normality and homogeneity of variances was verified to validate tests. When a significant overall difference was observed, pairwise differences between group means were evaluated using Scheffe's comparison. The statistical package software SAS was used for all analyses.

Results

Inactivated Virus Experiment (see Table 1)

Figure 1 shows the results of total and differential BALF cell counts obtained from mice sacrificed 3 weeks after Sendai inoculation. Saline-instilled animals show normal cell counts in the lavage, whereas mice infected with live Sendai still present a slight residual virus-specific inflammation; this difference was not statistically significant. Repeated SR applications induced an increase of BALF inflammatory cells ($p < 0.001$ compared with non-SR-instilled groups). In mice presensitized with SR, a live Sendai virus infection induced a marked potentiation of the inflammatory response to the antigen. However, BALF total and differential cell counts from mice inoculated with inactivated Sendai virus were similar to those obtained from control animals. Moreover, the inactivated virus did not affect the response to SR in antigen-challenged mice; BAL cells and differentials were similar between the two groups. The number of BAL cells was significantly higher in the SR + live Sendai compared with SR alone or SR + inactivated Sendai ($p < 0.001$). Similar results were obtained 3 months following the viral inoculations (Fig. 2). Here again, live Sendai virus enhanced the response to continuous SR applications ($p = 0.001$), whereas inactivated virus had no effect on the SR inflammatory response (p not significant).

Table 1. Summary of the study design for the inactivated virus experiment. Animals were instilled 3 days/week, and all sacrifices were done 4 days after the final treatment. On day 3 of the 3rd week all animals were instilled twice: SR or saline and saline alone or with live or inactivated virus according to their respective group. S, Sendai; IS, inactivated Sendai; SR, *S. rectivirgula;* Sal, saline.

Weeks	Group					
	Saline	S	IS	SR	SR + S	SR + IS
1–3	Sal	Sal	Sal	SR	SR	SR
3 (day 3)	Sal	S	IS	Sal	S	IS
4–6	Sal	Sal	Sal *Sacrifice*	SR	SR	SR
7–15	Sal	Sal	Sal *Sacrifice*	SR	SR	SR

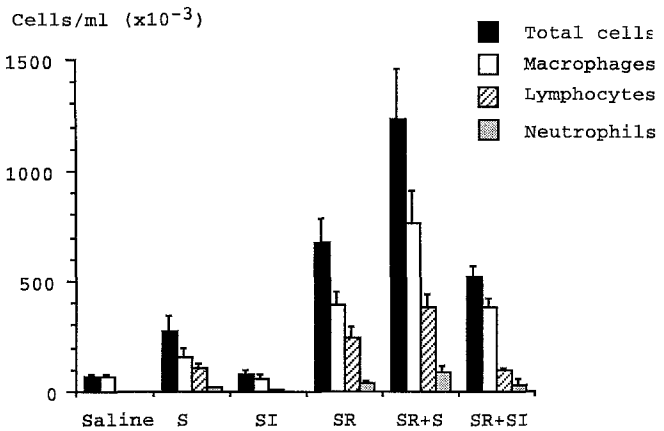

Fig. 1. Results of the BALF total and differential cell count for the six groups of mice sacrificed 3 weeks after live or inactivated Sendai virus administration in the appropriate groups. Results, expressed in the number of cells/ml of recovered BALF, are for five or six mice/group. As reported previously, animals who had a viral infection became hyperresponsive to FL antigen. Inactivated viral particles did not confer this enhancing effect. S, Sendai virus alone; SI, inactivated virus alone. SR, *S. rectivirgula* instillations alone, SR + S, both the antigen and the live virus; SR + SI, antigen and inactivated viruses. For the SR-treated groups, total BALF cells and individual cell types were significantly higher in the SR + S group, whereas BALF cells for the SR and SR + SI were similar.

Prechallenge Viral Infection (see Table 2)

Mice that recovered from the viral infection and were then instilled with SR antigens had a BALF inflammatory cellular response similar to that observed in the group of mice that received only the antigenic challenge. Results presented in Figure 3 were obtained after 1, 2, 3, and 4 weeks of SR treatment. The only statistically significant differences between these two groups was in the number of neutrophils, which was

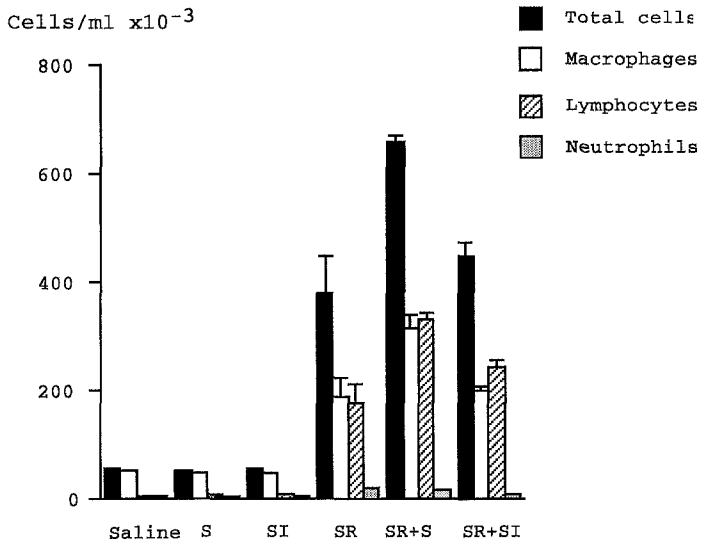

Fig. 2. Similar presentation as in Figure 1, except that the data were gathered 3 months after the virus instillation in the appropriate groups. Animals who received only saline, inactivated viruses, or live virus alone have normal BALF cells; those who received SR or SR + SI show a similar inflammation. The enhancing effect of a virus infection, which had occurred 3 months previously, was still present. The total BALF cell count of the SR + S group was significantly higher than for the SR or SR + SI groups. The latter two groups had similar BALF cell counts.

Table 2. Summary of the prechallenge viral infection protocol. All abbreviations and timing of saline and *S. rectivirgula* instillations are as in Table 1. The difference here is that the live viral instillation preceded the beginning of *S. rectivirgula* challanges by 4 weeks.

Weeks	Group			
	Saline	Sendai	SR	SR + Sendai
1 (day 1)	Sal	S	Sal	S
5	Sal	Sal *Sacrifice*	SR	SR
6	Sal	Sal *Sacrifice*	SR	SR
7	Sal	Sal *Sacrifice*	SR	SR
8	Sal	Sal Sacrifice	SR	SR

smaller in the SR alone group than in the animals preinfected by the Sendai virus. All data from the SR-instilled groups were highly significantly different from both control groups, all p values at <0.001.

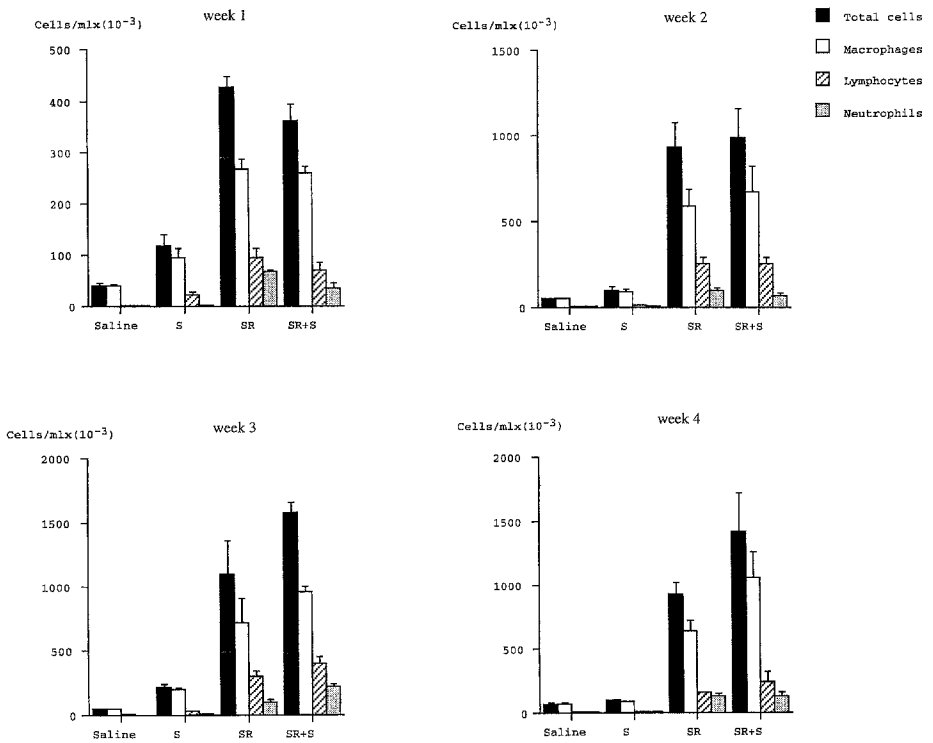

Fig. 3. BALF cells for mice sacrificed after 1, 2, 3, or 4 weeks of SR instillation which was started 4 weeks after a Sendai infection in the appropriate groups, saline in the others. The only significant difference between the SR + S and SR alone was a mild increase in neutrophils at 3 weeks. Overall, the virus-infected animals were not hyperresponsive to SR.

Discussion

This study further confirms the enhancing effect of a Sendai virus infection to subsequent challenges with the FL antigen SR in previously sensitized mice. In addition, the results show that the viral infection is required in the hypersensitization and that the virus must be given concomitantly with SR. The two experiments reported in this paper were done separately, therefore no direct comparisons between the two can be made.

The choice of giving the Sendai 28 days before the SR was arbitrary. This timing was chosen because it was sufficiently long to ensure that all inflammation caused by the viral infection would be gone and short enough to fall within the documented enhancing effect of the Sendai infection (6 months) [9].

The only variable evaluated in this study was the cellular response of the lungs as determined by BALF cell counts. Other variables such as lung histopathology or mediator release were not obtained. We believe that this simple outcome measurement was sufficient to answer the questions that prompted this study. For the prechallenge viral infection protocol no group was done to compare the effect of the viral infection

given simultaneously with SR. This was justified since the enhancing effect of Sendai virus when given simultaneously with SR has already clearly been demonstrated [9] and is confirmed further in the inactivated virus protocol of the current report.

Further studies will be needed to define how the viral infection is able to cause its enhancing effect. Several mechanisms of virus-induced exacerbation of the inflammatory response in our model could be hypothesized. Cumulative lung injuries following continuous exposures to SR antigens and a viral infection could lead to long term changes in the parenchymal structures and airway wall remodeling, leading to an increased influx of inflammatory cells. Alternatively, an increase in adherent-dependent migration of cells through the epithelial lining into the lumen of the respiratory tract could be involved. Tracheal epithelial cells infected with parainfluenza virus or rhinovirus have an enhanced expression of intercellular adhesion molecules (ICAM-1) [23]. Furthermore, ICAM-1 expression on epithelial cells is enhanced further in the presence of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1α (IL-α); or interferon-γ (IFN-γ) [4]. Both SR and Sendai virus have the capacity to induce the production of these proinflammatory cytokines [1, 10]. In our model, we have reported an increase in BALF levels of TNF-α and IL-1γ in SR + Sendai groups compared with SR alone groups 15 weeks after the Sendai virus infection [9]. We can therefore speculate that the viral infection and the cumulative effect of SR and Sendai induced the production of proinflammatory mediators which could provoke an enhanced expression of adhesion molecules, which could increase neutrophil influx into the inflammatory sites.

Sendai virus infection does not increase vascular permeability per se, but it does increase leak in the airways in response to neurokines, irritants, or other factors [17]. Perhaps such an increased vascular permeability and lung neutrophil recruitment in virus-infected mice could be achieved by SR antigen. One argument against this explanation, however, is that the potentiating effect on vascular leak and cell recruitment is present mostly in the airways, not in the alveoli [17]. Our model is one of interstitial inflammation with granuloma formation [9]. Another possibility is that the sensitization is caused by the lung inflammation of the viral infection. This hypothesis could be verified by using other means of producing an intercurrent lung inflammation by, for example, other viruses, endotoxins, β-glucans, or a bacterial infection.

Understanding the mechanisms involved in this model is important since it could help us understand what causes the hypersensitivity response to SR in subjects with FL.

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