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Viral Infection Modulates Expression of Hypersensitivity Pneumonitis¹

Gunnar Gudmundsson, Martha M. Monick, and Gary W. Hunninghake²

Hypersensitivity pneumonitis (HP) is a granulomatous, inflammatory lung disease caused by inhalation of organic Ags, most commonly thermophilic actinomycetes that cause farmer's lung disease. The early response to Ag is an increase in neutrophils in the lung, whereas the late response is a typical Th1-type granulomatous disease. Many patients who develop disease report a recent viral respiratory infection. These studies were undertaken to determine whether viruses can augment the inflammatory responses in HP. C57BL/6 mice were exposed to the thermophilic bacteria *Saccharopolyspora rectivirgula* (SR) for 3 consecutive days per wk for 3 wk. Some mice were exposed to SR at 2 wk after infection with respiratory syncytial virus (RSV), whereas others were exposed to SR after exposure to saline alone or to heat-inactivated RSV. SR-treated mice developed a typical, early neutrophil response and a late granulomatous inflammatory response. Up-regulation of IFN- γ and IL-2 gene expression was also found during the late response. These responses were augmented by recent RSV infection but not by heat-inactivated RSV. Mice with a previous RSV infection also had a greater early neutrophil response to SR, with increased macrophage inflammatory protein-2 (MIP-2, murine equivalent of IL-8) release in bronchoalveolar lavage fluid. These studies suggest that viral infection can augment both the early and late inflammatory responses in HP. *The Journal of Immunology*, 1999, 162: 7397–7401.

Hypersensitivity pneumonitis (HP)³ is a syndrome caused by repeated inhalation and sensitization to an organic Ag (1–6). At early timepoints after inhalation of Ag, there is an increase in neutrophils in the lung; at later timepoints, the lung disease is characterized by a classical Th1-type granulomatous disease. The most common Ags are thermophilic actinomycetes that cause farmer's lung disease, but other organic Ags can also cause HP (2). Only 10–15% of individuals exposed to these Ags will develop overt disease, because most of those that are exposed will be asymptomatic (3). Sometimes, a recent viral respiratory infection is described before the onset of HP, or with worsening symptoms of HP. These latter observations suggest that it is possible that a viral infection may predispose individuals to develop increased clinical manifestations of HP.

In prior studies, we used a well-described murine model to study HP. In this model, mice exposed to the actinomycete *Saccharopolyspora rectivirgula* (SR) (previously named *Micropolyspora faeni* and *Faeni rectivirgula*) via nasal inhalation develop diffuse

bronchoalveolitis and form granulomas in the lung (7–9). Using IFN- γ knockout mice, we showed that IFN- γ is necessary for granuloma formation in HP. Replacement of IFN- γ in the IFN- γ knockout mice resulted in an expression of HP in the lung that was similar to that of wild-type littermates (7). In another study, we showed that IL-12 was important for regulation of the expression of HP (8). The observations of these studies suggested that a Th1-type immune response might determine the expression of HP.

Respiratory infections are common, both in children and adults (10, 11). In mice, these infections can cause a Th1 immune response and an influx of neutrophils at sites of infection (12–15). This study was done to evaluate whether a previous respiratory tract infection could augment the inflammatory responses that are seen in a murine model of HP. Our study demonstrates that a previous respiratory syncytial virus (RSV) infection can augment the inflammatory responses in HP, most likely through the up-regulation of Th1 immune responses and the release of IL-8 and other chemoattractants by airway epithelium and inflammatory cells. Our study also shows that prior viral replication is necessary for this to occur.

Materials and Methods

Animals

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female mice weighing 18–24 g were used for these studies. They were housed in an Ag-free and virus-free environment at the University of Iowa Animal Care Unit and maintained on standard mouse chow and water ad libitum. All animal care and housing requirements set forth by the National Institutes of Health Committee on the Care and Use of Laboratory Animal Resources were followed, and animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Antigen

Ag was prepared from a strain of SR that had been obtained from the American Type Culture Collection (ATCC) (catalog no. 29034, Manassas, VA). It was grown in a trypticase soy broth in a 55°C shaking incubator for 4 days, centrifuged, and rinsed with distilled water three times. Next, the Ag was homogenized and lyophilized (17). Ag was resuspended in pyrogen-free saline. A *Limulus* amoebocyte lysate assay from Sigma (St. Louis, MO) showed that this material was endotoxin-free.

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³ Abbreviations used in this paper: HP, hypersensitivity pneumonitis; SR, *Saccharopolyspora rectivirgula*; RSV, respiratory syncytial virus; hRSV, heat-inactivated RSV; TCID₅₀, 50% tissue culture infective dose; BAL, bronchoalveolar lavage; MIP, macrophage inflammatory protein; RPA, RNase protection assay.

Virus

RSV (strain A2, lot 3W) was obtained from ATCC, where it was propagated in Hep-2 cells and harvested at a concentration of 10^6 50% tissue culture infective dose (TCID₅₀)/ml. TCID₅₀ refers to the quantity of virus that will produce obvious cytopathic effects in 50% of the tissue culture plates infected, and was calculated using the method of Reed and Muench (18). Sterile vials were kept frozen at -70°C . For all experiments, a new vial of virus was rapidly thawed at 37°C and used immediately. Heat inactivation of the virus was accomplished via incubation at 56°C for 2 h.

Plaque assays

To confirm infection with RSV, plaque assays were used as described previously (19). Briefly, 12-well tissue culture plates were seeded with Hep-2 cells at a concentration of 3.0×10^5 cells/well and incubated at 37°C . After 24 h, 0.1 ml of serial dilutions of RSV were added to each well and incubated at 37°C for 2 h. The cells were then washed twice with PBS, and 2 ml of a 1.2% methylcellulose overlay was added to each well. The cells were incubated at 37°C for 5 days, fixed with 10% formalin, and stained with 1% crystal violet. Next, plaques were analyzed using a dissecting microscope.

Induction of RSV infection and HP in mice

RSV infection was induced by instilling 10^5 TCID₅₀ of virus in 100 μl saline, intranasally, under light anesthesia (20). Other mice received a similar amount of heat-inactivated RSV (hRSV) or saline alone. The mice were then weighed and monitored daily for signs of illness for 2 wk. At 2 wk after the infection with RSV or hRSV, HP was induced by instilling 150 μg of SR Ag in saline intranasally under light anesthesia as described previously (7–9). This was done for 3 consecutive days per wk for 3 wk. Mice were sacrificed 4 days after the last exposure with pentobarbital injection.

Bronchoalveolar lavage (BAL)

After euthanasia, a 20-gauge catheter was inserted into the trachea. BAL samples were obtained by washing the lungs with three 1-ml aliquots of 0.9% saline. After centrifugation, BAL cell pellets were washed and resuspended in HBSS; total cell counts were enumerated using a Coulter counter (Coulter Electronics, Hialeah, FL). Cytospin preparations were fixed and stained using Diff-Quick staining (Baxter, McGaw Park, IL). Differential cell counts were made on 200 cells using standard morphologic criteria to identify the cells as neutrophils, eosinophils, lymphocytes, or macrophages (7–9).

Histologic evaluation

Lungs were perfused with 2% paraformaldehyde through the heart and trachea and fixed in 2% paraformaldehyde-PBS. The sections were embedded in paraffin, cut in 5- μm -thick sections, and stained with hematoxylin and eosin. The sections were evaluated by light microscopy. The slides were evaluated without knowledge of the type of exposure to Ag or virus. The lung fields were evaluated for extent of inflammation and granuloma formation, and this was expressed as a percentage of the total area of the lung fields. The area covered by an eyepiece grid (0.99×0.99 mm using $\times 100$ magnification) was judged to be normal or abnormal. An average of 200 fields was evaluated from each mouse as described previously (7–9). The results of the evaluation of the inflammatory changes were reproducible for two evaluations ($r = 0.88$).

RNA isolation and RNase protection assay (RPA)

Whole cell RNA was isolated by the protocol of Chirgwin et al. and Maniatis et al. (21, 22). The RNA was fractionated on a 1.5% agarose gel

Table I. Weights of C57BL/6 mice in grams^a

	Day 0	Day 7	Day 14
Control	18.3 \pm 0.6	19.0 \pm 0.7	19.8 \pm 0.9
RSV-treated	18.5 \pm 0.5	19.3 \pm 0.8	20.1 \pm 0.6
hRSV-treated	18.6 \pm 0.5	19.2 \pm 0.3	20.0 \pm 0.4

^a Mean weight in grams \pm SEM derived from eight individual mice per group. There was no statistical difference between the groups.

containing 2.2 M formaldehyde by the method of Lehrach et al. (23). The gels were stained with ethidium bromide and destained overnight in 0.1% ammonium acetate to assess RNA integrity. Measuring the ratio and absorbencies at 260 and 280 nm quantitated the yield and purity of the RNA. Gene transcripts were detected using an RPA (RiboQuant, MultiProbe RPA system, PharMingen, San Diego, CA) as described by the manufacturer. The probe that included DNA templates for cytokines (IL-2 and IFN- γ) and housekeeping proteins (GAPDH and L32) was used to generate antisense cRNA transcripts. A total of 10 μg of total RNA was hybridized with a ³²P-labeled antisense cRNA probe set in a solution hybridization buffer for 14 h at 56°C . The nonhybridized ssRNA was digested with a mixture of RNase A and T1. The remaining protected RNA fragments were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and were ethanol-precipitated. The protected hybridization products were separated on a 5% acrylamide/8 M urea gel. The gel was dried on a vacuum gel dryer at 80°C , wrapped in plastic wrap, and exposed to x-ray film overnight at -70°C to visualize the protected hybridized probe.

ELISA

IL-2 and macrophage inflammatory protein-2 (MIP-2) levels in BAL fluid were measured by ELISA (Genzyme, Cambridge, MA). The sensitivity of the IL-2 assay was 1.5 pg/ml. The sensitivity of the MIP-2 assay was 1.5 pg/ml.

Statistics

Statistical analysis was performed using a Student *t* test. For a comparison of histologic evaluations, Kruskal-Wallis one-way ANOVA by ranks was used. Values are expressed as mean \pm SEM. The 95% confidence limit was taken as significant ($p < 0.05$).

Results

Effect of viral infection alone

The C57BL/6 mice did not show any signs of illness during the first 2 wk after RSV infection, and there was no mortality. Weights were also similar between virus-infected mice and other groups of mice as shown in Table I. Virus infection in lungs from RSV-infected mice was demonstrated with a plaque assay at 5 days postinfection but not at 14 days postinfection. The histology of the lungs of RSV-infected mice was normal for ≤ 14 days postinfection (data not shown). RSV-infected mice had a 2-fold increase in total numbers of cells in BAL fluid at 7 days postinfection compared with the saline control group and the hRSV-treated group (Table II). At the 7-day timepoint, significantly larger numbers of the cells were lymphocytes in the RSV-infected group compared with the two other groups. By 14 days postinfection, there were no

Table II. Comparison of cells in BAL fluid from mice killed 7 or 14 days after RSV infection^a

	Total Cells	Macrophages	Neutrophils	Lymphocytes
Control day 7	2.70 \pm 0.27 ^b	2.70 \pm 0.00 (100) ^c	0.00 \pm 0.00 (0)	0.00 \pm 0.00 (0)
Control day 14	3.52 \pm 0.40	3.52 \pm 0.00 (100)	0.00 \pm 0.00 (0)	0.00 \pm 0.00 (0)
RSV day 7	7.12 \pm 0.70	5.74 \pm 0.70 (81)	0.30 \pm 0.30 (0.5)	1.35 \pm 0.06 (19) ^c
RSV day 14	3.47 \pm 0.30	3.40 \pm 0.30 (98)	0.00 \pm 0.00 (0)	0.07 \pm 0.02 (2)
hRSV day 7	3.19 \pm 0.29	3.07 \pm 0.30 (97)	0.00 \pm 0.00 (0)	0.09 \pm 0.03 (3)
hRSV day 14	3.24 \pm 0.28	3.14 \pm 0.40 (97)	0.00 \pm 0.00 (0)	+0.03 (3)

^a $p < 0.01$ compared with hRSV and control group.

^b Mean number of cells $\times 10^5 \pm$ SEM derived from four individual mice per group.

^c Mean percentage of cells.

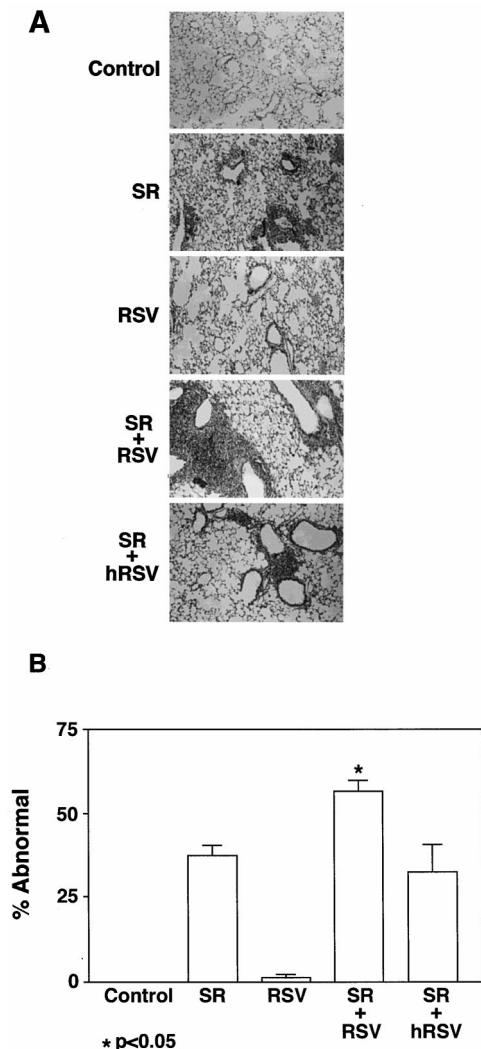


FIGURE 1. RSV increases expression of HP in mice exposed to SR Ag. C57BL/6 mice were exposed to RSV (hRSV) or saline followed by SR or saline as described in *Materials and Methods*. *A*, Animals were sacrificed, and lung pathology was obtained (hematoxylin and eosin-stained histology sections) ($\times 100$ magnification). *B*, Histologic scores were obtained for each animal, and statistical analysis was performed. The percentage of abnormal lung tissue is shown on the ordinate, and types of exposure are indicated on the abscissa. Data are expressed as the mean \pm SEM for eight animals in each group. Statistical analysis was performed using Kruskal-Wallis one-way ANOVA by ranks.

differences in the numbers of any of the types of cells in BAL fluid in RSV-infected mice compared with the other control groups. These observations suggest that the C57BL/6 mice respond to RSV infection with lymphocytic alveolitis, but without signs of illness.

Effect of RSV on the late granulomatous-type response to SR

C57BL/6 mice that were not exposed to SR did not develop any histologic abnormalities, as shown in Fig. 1. This was the case both for the mice infected with RSV and for the mice not infected with RSV. C57BL/6 mice exposed to SR but not infected with RSV developed a granulomatous inflammatory response that was most prominent around small airways. Mice that were first infected with RSV and subsequently exposed to SR had a significantly greater granulomatous inflammatory response compared with mice exposed to SR alone. Mice that were treated with hRSV and subse-

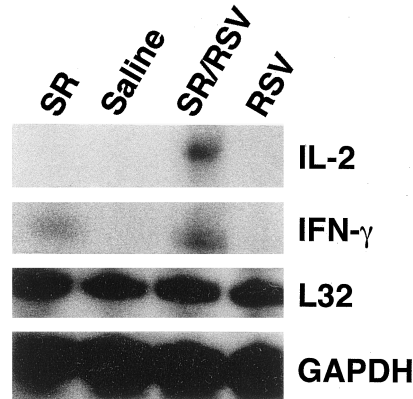


FIGURE 2. RSV increases cytokine (IFN- γ and IL-2) mRNA levels in lungs exposed to SR Ag. C57BL/6 mice were treated with RSV or saline followed by SR or saline. Animals were sacrificed 4 days after the final SR exposure, and whole cell RNA was obtained from lung tissue. RNA levels were determined using an RPA as described in *Materials and Methods*. An autoradiograph of 32 P-labeled mRNA for IFN- γ , IL-2, and the housekeeping genes L32 and GAPDH is shown on the figure ($n = 3$).

quently with SR had inflammatory changes that were similar to mice that were treated with SR alone. These observations suggest that C57BL/6 mice develop a more severe granulomatous inflammatory response to SR if they have been exposed previously to RSV infection, even though the viral infection has resolved at the time of exposure to SR. Prior viral infection was necessary for the increased severity of the granulomatous response, because mice infected with heat-inactivated virus did not have a more severe inflammatory response. At the time of the late response to SR, C57BL/6 mice demonstrated an up-regulation of IL-2 and IFN- γ gene expression (Fig. 2). Mice infected with RSV before they were exposed to SR had a greater increase in expression of these genes. IL-2 was also significantly increased in BAL fluid (Fig. 3). These studies show that RSV infection accentuates the Th1 response to SR.

Effect of RSV on the early neutrophil-type response to SR

We evaluated whether RSV might up-regulate the early neutrophil-type response to SR. For these studies, mice were killed on day 14 after viral installation and 4 h after a single dose of SR.

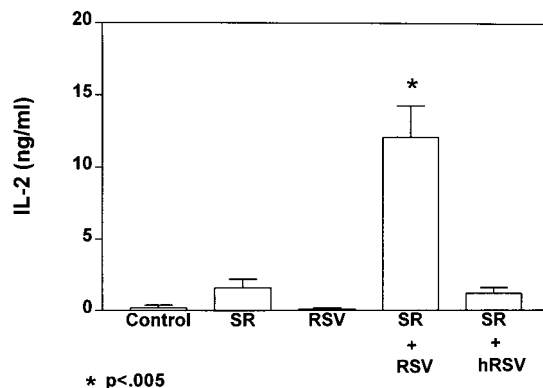


FIGURE 3. RSV increases the amount of IL-2 found in BAL fluid in mice treated with SR Ag. C57BL/6 mice were treated with RSV, hRSV, or saline followed by SR or saline. The mice were sacrificed 4 days after the last SR exposure, and BAL fluid was collected. IL-2 levels were determined by ELISA. A quantitation of IL-2 is shown on the ordinate; types of exposures are indicated on the abscissa. Data are expressed as the mean \pm SEM for four animals in each group. Statistical analysis was performed using a Student *t* test.

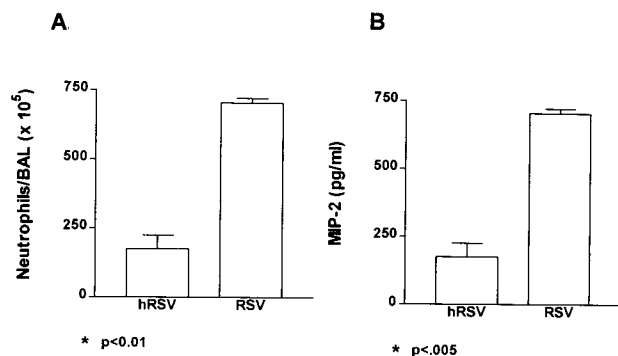


FIGURE 4. RSV increases the numbers of neutrophils and amounts of MIP-2 found in the lungs of mice after acute exposure to SR Ag. C57BL/6 mice were exposed to RSV or hRSV. After 14 days, this exposure was followed by an intranasal exposure of a single dose of SR. Animals were sacrificed at 4 h after SR exposure, and BAL was performed. *A*, Numbers of neutrophils present in BAL in RSV + SR and hRSV + SR animals ($n = 3$). *B*, MIP-2 levels were quantitated in the BAL fluid ($n = 3$). Data are expressed as the mean \pm SEM. Statistical analysis was performed using a Student *t* test.

Mice that were treated previously with live RSV had a greater neutrophil response compared with those treated with hRSV (Fig. 4A). The RSV-infected mice also had significantly more MIP-2 (the murine equivalent of IL-8) in the BAL fluid, as demonstrated in Fig. 4B. These observations suggest that viral infection also augments the neutrophil response to SR.

Discussion

HP is characterized by an early, neutrophil-type response and a later Th1-type granulomatous response to inhaled Ags. This study shows that a recent respiratory viral infection can augment both of these inflammatory responses in HP. In these studies, mice were exposed to SR Ag after they had recovered from an RSV infection. Mice that were infected previously with RSV had a greater early neutrophil response and a greater granulomatous inflammatory response to SR than mice that were not infected with virus. Mice exposed to heat-inactivated virus were similar to control mice, suggesting that live virus is necessary for this effect. Our studies add important information to prior studies. First, they provide the first histologic evidence that a viral infection can augment the late granulomatous response in HP. They also show that a virus can prime the lung for a greater early neutrophil-type response to Ag. These studies are consistent with clinical observations that suggest that in some patients, prior viral infections may enhance the clinical expression of HP. In other patients, it is possible that the syndrome is caused by the initial response to the thermophilic bacteria.

Previous studies from our laboratory have shown that both SR and RSV can stimulate IL-8 release from respiratory epithelial cells (24, 25). IL-8 is an important chemoattractant for neutrophils, which are prominent in BAL in early HP (6). This may explain, at least in part, how viral infection augments the early inflammatory events in HP. This hypothesis was supported, in these studies, by the observation that when mice are given a single dose of SR after recovery from RSV infection, they have a greater early neutrophil response than mice without a preceding virus infection. In addition, there is greater release of MIP-2 (the mouse equivalent of IL-8) in BAL fluid. It is also likely that other chemoattractants from both epithelial cells and inflammatory cells were involved.

Previous studies using Sendai viral infection in mice showed that a concomitant viral infection and exposure to SR or a viral

infection after SR exposure resulted in an increase in total cell counts in BAL fluid. There were increases in macrophages, lymphocytes, and neutrophils. These changes were noted for ≤ 30 wk after Sendai virus infection. Histologic evaluation showed either no increase in inflammatory responses or a tendency toward more granuloma formation. The levels of TNF- α and IL-1 α in BAL fluid were greater in mice with exposures both to Sendai virus and SR compared with mice exposed to SR alone. Mice infected with Sendai virus before exposures to SR had no augmentation in lung responses, nor did mice that were given inactivated virus (27–30). Although these studies did not document an increase in the histologic expressions of HP in response to viral infection, the Sendai virus studies are consistent with the results of this study. These observations may also suggest that not all viral infections have the same effect in HP.

Th1 and Th2 subsets of T cells are defined on the basis of their pattern of production of cytokines (31). Th1 cytokines include IL-2, IL-12, and IFN- γ . Th2 cytokines include IL-10, IL-4, and IL-5. In a murine model of experimental HP, Schuyler et al. showed that Th1 cells were able to adoptively transfer HP (32). We have shown previously that IFN- γ is necessary for the pathogenesis of HP (7). When mice that do not produce IFN- γ are exposed to Ag, they do not develop granulomatous inflammation. Further, we showed that IL-12, which augments the release of IFN- γ in response to Ag, plays an important role in modifying the expression of HP (8). In addition, IL-10, which dampens the expression of IFN- γ , also limits the development of HP (9). The present study shows that a prior viral infection augments the Th1-type response to Ag in HP.

Several studies have demonstrated the importance of Th1 responses in murine RSV infections. When mice are primed with live virus, a Th1 response with increases in IL-2 and IFN- γ , in addition to a decrease in IL-4 is observed (15). A study by Schwarze et al. showed that BALB/c mice that were infected with RSV developed a Th1 response in peribronchial lymph node cells in vitro. If these animals were then sensitized to OVA via the airways, they developed a Th2 response (12). Studies on mice infected with Sendai virus have also demonstrated that a Th1 response occurs (35).

Although most studies on RSV infections in mice have been done in BALB/c mice, we chose to use C57BL/6 mice. These mice have been used for most studies of murine HP, especially with virus infections (27–30). C57BL/6 mice are also fairly resistant to RSV infections. Previous studies have shown that, at the dose used in our studies, mice do not develop any sign of infection, such as weight loss, raised fur, or lethargy (20). This was confirmed again in our studies. Thus, it is unlikely that the differences between SR-treated or SR-and RSV-treated groups could have been from malnutrition, which is known to alter the expression of granulomatous diseases (36, 37).

Taken together, these studies show that a previous viral infection can augment both the early and the late inflammatory responses in a murine model of HP. These studies may provide insight into how individual susceptibility to HP can vary and how important avoidance of Ag exposure may be after a viral respiratory infection.

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