

# Rabies Superantigen as a V $\beta$ T-dependent Adjuvant

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## Summary

Recently we reported evidence that the nucleocapsid (NC) of rabies virus is a V $\beta$ 8-specific exogenous superantigen (SAg) in humans and a V $\beta$ 6-specific SAg in BALB/c mice. NC was also found to stimulate rabies vaccination by enhancing the rabies neutralizing antibody response. In this study, we tested the hypothesis that the stimulating effect of NC and its SAg properties are linked. To do this, we studied the effect of rabies SAg on the immune response to an unrelated antigen, the influenza virus, and compared the response in two congenic strains of mice, BALB/c and BALB/D2. BALB/c mice are rabies SAg responsive, whereas BALB/D2 mice are not responsive to SAg activation by rabies NC because they lack the SAg recognition element, the V $\beta$ 6 T cell receptor. In BALB/c mice, coinjection of rabies SAg with inactivated influenza virus resulted in a rapid and long-term increase in (a) the titres of influenza virus-specific antibodies (IgG and IgM), including protective hemagglutination-inhibiting antibodies, (b) antigen-specific proliferation and, (c) IL-2 and IL-4 secretion by lymph node lymphocytes, when compared to mice that received influenza virus only. In contrast, in BALB/D2 mice, neither antibody nor lymphocyte responses were stimulated. Moreover, during establishment of the primary response, the increase in influenza-primed T cells was mainly restricted to those bearing a V $\beta$ 6 TCR. These data establish that rabies SAg can stimulate both T and B cell-specific responses to an unrelated antigen, depending on expression of the SAg target (V $\beta$ 6 T lymphocytes). This is the first report linking NC adjuvant properties with its SAg mechanism.

The molecular basis of antigen-specific T lymphocyte activation is the recognition of a specific peptide antigen by the  $\alpha\beta$  chains of the T cell receptor (TCR). To be recognized by the TCR, antigens first need to be cleaved by the antigen-presenting cells (APC) into small peptides before being associated with the major histocompatibility complex (MHC) molecules through their peptide-binding groove and presented on the surface of APC. Specific recognition of the peptide-MHC complex by the TCR is generated by somatic recombination of the variable elements of the TCR  $\alpha$  and  $\beta$  chains. As a consequence, the frequency of T cells activated by one peptide is extremely low (1 in  $10^4$  to  $10^6$ ). Superantigens (SAg)<sup>1</sup> are a special class of antigens that, unlike peptides, bind outside the peptide groove, and cross-link the outer faces of the MHC class II molecules and the variable  $\beta$  (V $\beta$ ) chains of the TCR (1). SAg are recognized almost without MHC restriction. They do not require antigen processing and target specifically one or several of the 20 or so V $\beta$  family T lymphocytes. Thus, the frequency of a T cell response to a SAg

is extremely high (up to 1 in  $10^2$ ). SAg activation is characterized by a sudden expansion of the T cells bearing the correct V $\beta$ , and secretion of large amounts of cytokines. When the APC are B lymphocytes, the molecular bridge formed by the SAg between T and B lymphocytes may induce unusual T-B interactions and trigger B cells to produce antibodies. When T and/or B lymphocytes are already engaged in an antigen-specific response, it can be assumed that SAg could amplify the antigen-specific response either directly because of the T-B interaction or indirectly because of cytokine secretion (2). Theoretically, one could exploit the SAg ability to enhance an associated immune response and use SAg as an adjuvant.

SAg are encoded not only by bacteria or mycoplasma but also by viruses, in particular exogenous and endogenous mouse mammary tumor retroviruses (MMTV) and most recently, the rabies virus (3, 4). With regard to the rabies virus, we have already established that the viral nucleocapsid (NC), and its main component the N protein, behave as V $\beta$ 8-specific exogenous SAg in humans (4, 5) and as V $\beta$ 6-specific SAg in BALB/c mice but not in congenic BALB/D2 mice, which lack the V $\beta$ 6 T cells responsive to rabies SAg (6). Additionally, the NC can induce cognate T-B interactions in vitro since, in human tonsillar cells, we found that the NC triggers T lymphocytes to secrete cy-

<sup>1</sup>Abbreviations used in this paper: HA, hemagglutinin; HI, Hemagglutination inhibition; HIU, HI unit; LNC, lymph node cells; MAM, *Mycoplasma arthritidis* mitogen; MMTV, mouse mammary tumor virus; NC, nucleocapsid; SAg, superantigen.

tokines and B lymphocytes to produce IgG, the latter only in the presence of T cells (5). Previous studies established that NC could enhance the immune response to rabies vaccination or to an unrelated peptide (7, 8). We hypothesized that rabies SAg would stimulate the immune response against an unrelated vaccinal antigen and could therefore be used as an adjuvant. In this study, we tested whether rabies SAg stimulated the immune response to influenza in two congenic strains of mice, BALB/c and BALB/D2, which differ only in their ability to respond to rabies SAg.

Our results show that the rabies NC does in fact stimulate an antibody response directed against a simultaneously injected non-rabies antigen, and that its SAg properties are responsible of the NC adjuvant capacity. This report is the first direct evidence establishing that a SAg can be used successfully as an adjuvant, and the first description of an explanatory mechanism.

## Materials and Methods

**Reagents, Virus, Cells, and Culture Medium.** NC was prepared from rabies virus-infected hamster kidney cell (mycoplasma free) lysates and partially purified through CsCl gradients as previously described (9).

Inactivated influenza virus (310  $\mu$ g HA/ml), purified influenza virus (8 mg/ml) used for ELISA and for in vitro lymphocyte proliferation, and hyperimmune rabbit serum (16,000–32,000 HIU/ml), were prepared with the influenza N1B16 virus, A-H1N1 strain, a hybrid between X31 and A/Taiwan/1/86 (gifts from Pasteur Mérieux Sérums et Vaccins, Marcy l'Etoile, France). Rat anti-mouse IL-4 mAb (capture antibodies BVD4-1D11 and biotinylated detection antibodies BVD6-24G2), and biotinylated rat anti-mouse  $\text{V}\beta$ 6 and  $\text{V}\beta$ 10 mAbs (RR4-7 and B21.5) were purchased from PharMingen (San Diego, CA). HRP-conjugated streptavidin and biotin-conjugated sheep anti-mouse whole Ig were purchased from Amersham (Amersham, UK). 2,2'-azino-di-[3-ethyl-benzthiazolin-sulfonate-6] (ABTS) was obtained from Boehringer Mannheim (Indianapolis, IN). [ $^3\text{H}$ ]Thymidine was purchased from Dupont de Nemours (Les Ulis, France). Receptor destroying enzyme (RDE) (cholera vibrio neuraminidase) was purchased from Sigma Chemical Co. (St. Louis, MO).

Lymphocytes from popliteal lymph nodes (LN) were cultured in RPMI 1640 (GIBCO BRL, Cergy-Pontoise, France), 2 mM L-glutamine,  $10^{-2}$  mM  $\beta$ -mercaptoethanol and 100 U/ml gentamicin, supplemented with 10% fetal calf serum. The IL-2-dependent CTLL-2 cell line (10) was maintained in the same medium supplemented with 10% condition medium of ConA-stimulated rat splenocytes.

**Mice.** Two types of mice were used in these experiments: female BALB/c (H-2<sup>d</sup>, I-E+, and  $\text{V}\beta$ 3-,  $\text{V}\beta$ 5-, and  $\text{V}\beta$ 11- deleted) purchased from Janvier (St-Berthevin, France) and congenic BALB/D2 (H-2<sup>d</sup>, I-E+, and  $\text{V}\beta$ 3-,  $\text{V}\beta$ 5-,  $\text{V}\beta$ 11-,  $\text{V}\beta$ 6-,  $\text{V}\beta$ 7-,  $\text{V}\beta$ 8.1-, and  $\text{V}\beta$ 9- deleted), a gift from Martine Brulay-Rosset (Villejuif, France).

**Immunization of Mice.** To measure the putative rabies SAg adjuvant effect on influenza immunization, 8-wk-old BALB/c or BALB/D2 mice were injected i.p. with 100  $\mu$ l of inactivated influenza virus containing 5  $\mu$ g HA alone or with 20  $\mu$ g of SAg. A secondary immunization was given by the same route with 2  $\mu$ g HA 3 mo (= 90 d) after priming (see Fig. 2). Blood was taken from the retro-orbital-sinus every week for 12 wk after the prim-

ing and for 3 wk after each secondary immunization. For in vitro proliferation assays and cytokine production, BALB/c and BALB/D2 mice were injected in the footpad with 50  $\mu$ l of inactivated influenza virus alone (5  $\mu$ g HA) or with influenza virus and SAg (20  $\mu$ g). Control mice were injected with 50  $\mu$ l pyrogen-free saline solution or SAg alone. Mice were killed after 1, 3, 5, or 7 d, popliteal LN were removed aseptically, and LN cells (LNC) prepared for culture assays.

**Ig Production.** Anti-influenza and anti-NC antibody concentrations were determined from mice sera by ELISA. Briefly, purified influenza virus or NC (200 ng/well) was coated overnight on Maxisorb Immunolon plates (Nunc, Roskilde, Denmark) in 0.05 M carbonate buffer, pH 9.6, at 4°C. After blocking with 10% normal serum (rabbit, goat, or sheep according to the species of the detection antibody) in phosphate buffered saline (PBS) with  $\text{Ca}^{2+}$   $\text{Mg}^{2+}$  containing 0.05% Tween 20 (PBS-Tween), serial threefold dilutions of serum, starting with a 1/300 dilution, were incubated at 37°C for 90 min. Plates were washed with PBS-Tween, and then incubated with biotin-conjugated anti-mouse whole Ig. Biotinylated anti-mouse Ig were detected with streptavidin-peroxidase conjugate and then ABTS. Optical densities were measured at 405 nm using a photometer. Results were expressed in arbitrary units/ml (AU/ml) using serum of hyperimmunized mice as reference for influenza-specific antibodies and a NC-specific mAb, PVA-3, (9), for NC-specific antibodies.

**Hemagglutination Inhibition Antibody Titrations.** Sera were prepared with RDE for 18 h at 37°C and with hen erythrocytes for 30 min at room temperature to eliminate nonspecific inhibitors of hemagglutination. Hemagglutination inhibition (HI) titrations were performed in 96-well round-bottom polystyrene microtiter plates. Serial twofold dilutions of mouse sera in 50  $\mu$ l of PBS, pH 7.3, were mixed with four hemagglutination units of virus in 50  $\mu$ l of PBS and incubated for 60 min at room temperature. A 0.5% (vol/vol) hen erythrocyte suspension in PBS was added to the virus-antibody mixture in a volume of 50  $\mu$ l, and plates were incubated for 60 min at room temperature. Rabbit influenza-specific serum was used as a standard. HI antibody titers were expressed in HI unit per ml (HIU/ml) as the reciprocal of the highest serum dilution inhibiting four hemagglutination units of virus.

**Limiting Dilution Assay of Purified  $\text{V}\beta$  T Cells.**  $\text{V}\beta$ 6 or  $\text{V}\beta$ 10 T lymphocytes were purified from the popliteal LN of mice injected 7 d before with SAg + influenza or influenza alone. LNC were incubated 30 min on ice with biotin-conjugated anti- $\text{V}\beta$ 6 or anti- $\text{V}\beta$ 10 mAb in PBS, 5 mM EDTA, 1% BSA, washed and then incubated 15 min with streptavidin-coated magnetic microbeads (MACS; Miltenyi Biotech, Gladbach, Germany). Cells linked to the beads were firstly retained in a column through a powerful magnetic field (VarioMACS; Miltenyi Biotech) and then washed out. Harvested cells were over 95%  $\text{V}\beta$ 6 or  $\text{V}\beta$ 10 positive as assayed by cytofluorimetry analysis. Different concentrations of purified  $\text{V}\beta$  T cells ( $120 \times 10^3$  cells/well to  $2 \times 10^3$  cells/well) were distributed in 100  $\mu$ l of medium in wells of 96-well microtiter plate containing 50  $\mu$ l of irradiated (3,000 rad) splenocytes of nonimmunized BALB/c used as feeders ( $5 \times 10^5$  cells/well) and 50  $\mu$ l of influenza antigen (final concentration of 2  $\mu$ g/ml). 20 replicates were seeded for each concentration of cells and controls. Controls consisted of feeder cells alone cultivated with influenza antigen. The 24 h supernatants were assayed for IL-2 production by CTLL-2 proliferation. Wells were considered positive when counted cpm were three times higher than the average cpm counted in controls. Frequency of influenza-responsive cells were estimated by regressing (least square method) the log of the percentage of non-responder wells against the cell

number/well. Responder cell frequencies, their standard errors and ranges (confidence interval 95%) were then estimated using the reciprocal of the cell number corresponding to 37% of non-responder wells (11). Differences between frequencies of each treatment group were assessed by a one way variance analysis test (ANOVA).

**Cell Proliferation.** LNC at  $2 \times 10^5$  cells/well in a final volume of 200  $\mu$ l were cultured in 96-well plates at 37°C in 5% CO<sub>2</sub>, alone or with serial dilutions of anti-CD3 antibody or UV-inactivated influenza virus (0.01–10  $\mu$ g/ml). After 72 h, the cells were pulsed with 1  $\mu$ Ci/well of [<sup>3</sup>H]thymidine and harvested 8 h later with a Mach III harvester (Wallac, Turku, Finland). Radioactivity (cpm) was measured using a Micro- $\beta$  6-detector PLUS (Wallac).

**Interleukin Assays.** IL-2, and IL-4 productions were measured in 50  $\mu$ l of supernatants of either total LNC or V $\beta$  T purified cultures. IL-2 was measured in 24-h supernatants by a biological assay using IL-2-dependent CTLL-2 cell lines. Results were expressed in cpm, representing [<sup>3</sup>H]thymidine incorporation by 10<sup>4</sup> CTLL-2 cells cultured 24 h in the presence of supernatants. In each test, serial dilution of an IL-2 reference were assayed as control. IL-4 was measured in 48-h supernatants, by capture ELISA. Briefly, capture anti-mouse IL-4 rat antibody was coated overnight on Maxisorb Immunolon plates in 0.05 M carbonate buffer, pH 9.6, at 4°C. After blocking with 10% normal rat serum in PBS-Tween, LNC supernatants were incubated at 4°C overnight. Plates were washed with PBS-Tween, and then incubated with biotin-conjugated-detection anti-mouse IL-4 rat mAb, which was revealed with streptavidin-peroxidase conjugate and ABTS. Optical densities were measured at 405 nm. Cytokines concentrations were calculated using the linear portion of the curve obtained with recombinant standards run on each ELISA plate and expressed in AU/ml.

**Statistical Analysis.** Differences between groups were analyzed using Student's *t*-test and Mann-Whitney test. Equivalence between treatments was determined by correlation analysis. Significance was admitted at *P* < 0.05.

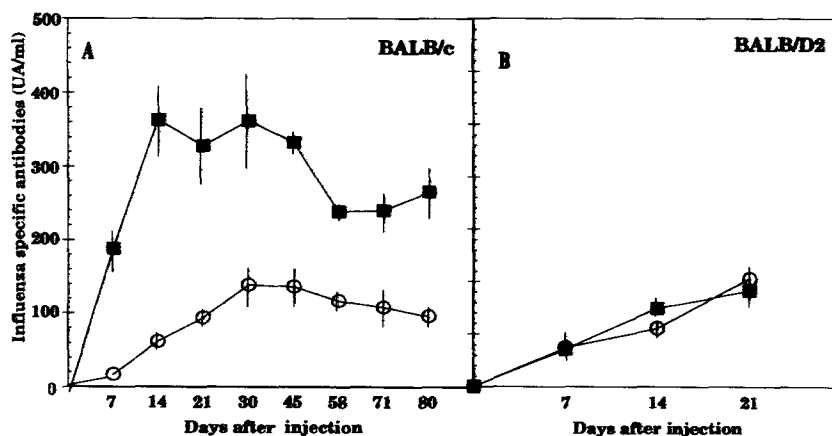
## Results

**Rabies SAg Induces a Long-term Increase of the Influenza-specific Antibody Response, in BALB/c (V $\beta$ 6+) but Not in BALB/D2 (V $\beta$ 6-) Mice.** Rabies SAg functions as a SAg in BALB/c mice, activating T lymphocytes bearing a V $\beta$ 6

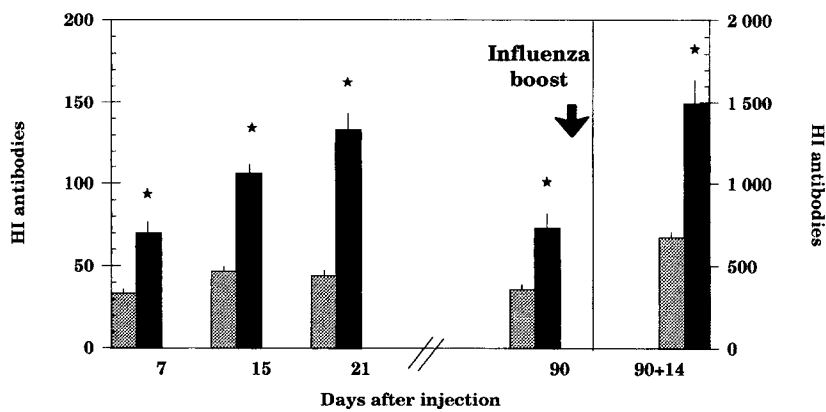
chain, but not in congenic BALB/D2 lacking V $\beta$ 6 T lymphocytes (6). The effect of rabies SAg on the antibody response to influenza immunization was compared in BALB/c (V $\beta$ 6+) and BALB/D2 (V $\beta$ 6-) mice. For both strains of mice, the influenza-specific whole antibody response was followed in two experimental groups. One group received a single injection of inactivated influenza virus (influenza-injected mice), the second group received the same dose of inactivated influenza virus mixed with rabies SAg (SAg + influenza-injected mice).

As shown in panel A of Fig. 1, rabies SAg had a stimulatory effect on the influenza-specific antibody response in BALB/c mice. This effect was very rapid, occurring during the first week. The antibody response plateau was reached 2 wk sooner in SAg + influenza-injected mice (d14) than in influenza-injected mice (d30). The stimulatory effect was long-lasting, since the whole antibody response in SAg + influenza-injected mice remained 10- to 3-fold higher than that observed in influenza-injected mice during the 80-d observation period. In contrast, as shown in the panel B of Fig. 1, SAg treatment did not significantly enhance the influenza-specific antibody response in BALB/D2 mice, as expected. Analysis of the anti-influenza IgG and IgM (data not shown) did not yield any difference either. BALB/D2 mice developed an influenza-specific antibody response comparable to the BALB/c one, and had been properly injected with SAg, since NC-specific antibodies developed (data not shown). These results suggest that rabies SAg adjuvant effect is not dependent upon antigenic recognition of rabies-NC or direct activation of B lymphocytes. On the contrary, they strongly support the hypothesis that V $\beta$  T cell-dependent SAg recognition plays a key role.

Previous studies indicated that mineral or vegetal adjuvants can amplify preferentially, if not exclusively, IgG1 or IgG2a antibodies (12, 13). We investigated whether rabies SAg modified the pattern of IgG isotypes or the Ig subclasses of the influenza-antibody responses. The distribution of IgM, IgG1, IgG2a, IgG2b, and whole IgG of influenza-specific antibodies were measured in the serum of rabies SAg + influenza injected and influenza-injected mice (data not shown). Rabies SAg stimulated all the iso-



**Figure 1.** A single injection of rabies SAg concomitantly with influenza antigen induces a very early, long-term increase of influenza-specific antibody response in BALB/c mice but has no effect in BALB/D2 mice. Effect of SAg on the whole antibody response to influenza in (A) BALB/c mice and (B) in BALB/D2 mice, lacking T lymphocytes expressing the SAg recognition element (V $\beta$ 6). Influenza-specific antibody titers, expressed in AU/ml, were measured every week, during 3 mo for BALB/c mice and 3 wk for BALB/D2 mice, in serum from mice receiving either SAg + influenza (■) or influenza alone (○) at day 0. Data presented are the mean titers  $\pm$  SEM of 4–6 mice per group.



**Figure 2.** Adjuvant effect of rabies SAg on secondary influenza-specific protective antibody response. Comparison of the protective HI antibodies response in BALB/c mice having received at day 0 influenza alone (gray bars) or SAg + influenza (black bars), followed by a secondary injection for all mice with influenza on day 90 (Influenza boost). HI antibodies titers, expressed in HIU/ml, measured for 3 mo after primary injection are presented on the left part of the scheme (left scale 0–200 HIU/ml), and those measured 2 wk after a boost of influenza alone are presented on the right part (right scale 0–2,000 HIU/ml). Data presented are the mean titers  $\pm$  SEM of 4–6 mice per group. An asterisk indicates significant difference, at  $P < 0.05$ , found between the two groups of mice.

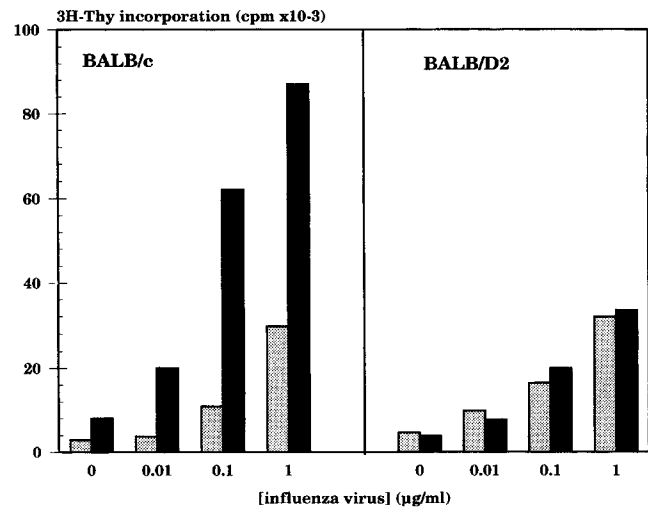
types with a slightly higher increase of the IgG1 antibodies. Therefore, rabies SAg does not seem to preferentially activate any particular class or isotype of influenza-specific antibodies, and instead seems to amplify the pattern of isotypes and subclasses already skewed by the influenza vaccination.

The adjuvant effect of rabies SAg was found to be dose dependent and to be affected by the immunization schedule (data not shown). For example, the same dose of SAg that enhanced antibody response when given simultaneously with the influenza virus failed to significantly enhance the antibody response when given 3 d before immunization (data not shown). However, SAg given 3 d before the influenza virus was efficient if the concentration was 10-fold higher.

*The Secondary Immune Response to Influenza Is Increased in Mice Primed with Influenza and Rabies SAg.* Hemagglutinin (HA) is a highly immunogenic surface protein of the influenza virus. It is the major antigen inducing antibodies which inhibit hemagglutination and neutralize the infectivity of the virus. Good correlation is observed between protection against influenza and the titers of hemagglutination-inhibiting antibodies (HI antibodies). The time course of the HI antibody concentration was compared in SAg + influenza- and influenza-injected mice at 7, 15, 21, and 90 d after immunization. At 90 d, mice from the two groups were boosted with influenza alone. Serum collected 14 d after the boost was analyzed for HI antibodies (Fig. 2). Rabies SAg-treated mice showed significantly greater titers of HI antibodies during the observation period with a peak on d21, indicating that influenza immunization associated with the rabies SAg has a greater protective capacity. Moreover, this twofold increase in HI antibodies observed between the two groups was maintained after a boost of influenza alone on day 90. These results show that rabies SAg can increase antibody level not only in a primary but also in a secondary response to an associated antigen. They also indicate that the rabies SAg adjuvant effect on the antibody response is a long-lasting one, since improvement of the secondary response can be obtained without a second injection of SAg. Taken together, these observations clearly establish that rabies SAg is an early and long-lasting immu-

nostimulant of the influenza antibody response especially, the protective response.

A boost with SAg alone was also performed to test whether SAg adjuvant can recruit antigen-specific antibodies in the absence of recall antigen. 3 mo after the secondary influenza injection, both groups of mice were injected with SAg alone. NC-specific and influenza-specific antibodies were measured for 3 wk after the secondary SAg injection (data not shown). No increase in influenza-specific antibodies could be observed. The last result indicates that the adjuvant effect requires the coadministration of SAg



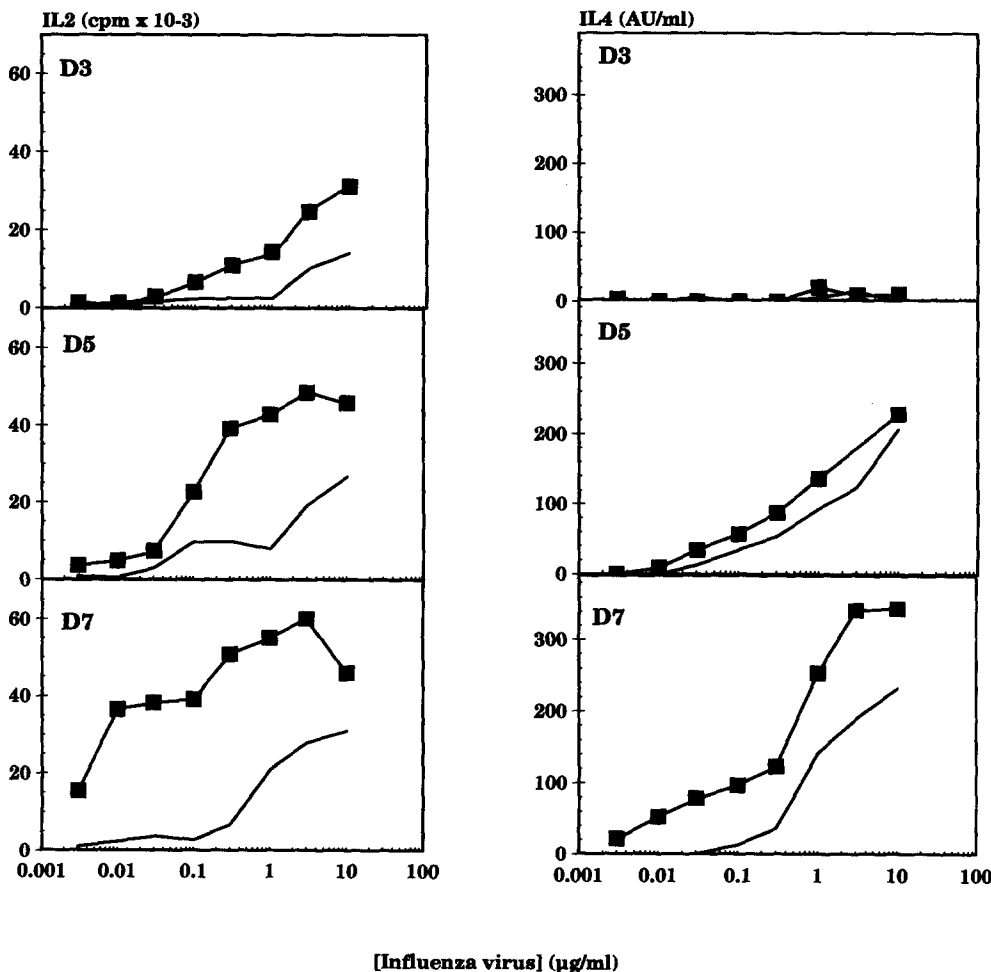
**Figure 3.** In vivo rabies SAg treatment enhances both spontaneous and influenza-specific proliferation of LNC from BALB/c mice but not BALB/D2 mice. Spontaneous proliferation obtained in cultures without stimulus ( $= 0 \mu\text{g/ml}$  of influenza virus) and influenza-specific proliferation obtained in cultures with 0.001–1  $\mu\text{g/ml}$  of inactivated influenza virus were measured in LNC collected from BALB/c mice (left panels) or from BALB/D2 mice (right panels) 3 d after receiving either influenza alone (gray bars) or SAg + influenza (black bars). Results expressed in  $\text{cpm} \times 10^{-3}$  were obtained with pooled LNC from two mice in each group and are representative of two separate experiments. A similar pattern was observed 5 and 7 d after injection. Thymidine incorporation in LNC cultures from mice injected with PBS or SAg alone did not exceed  $1 \times 10^3$  cpm.

with antigen and also confirms that rabies SAg has no cross-reactive epitopes with influenza virus antigens.

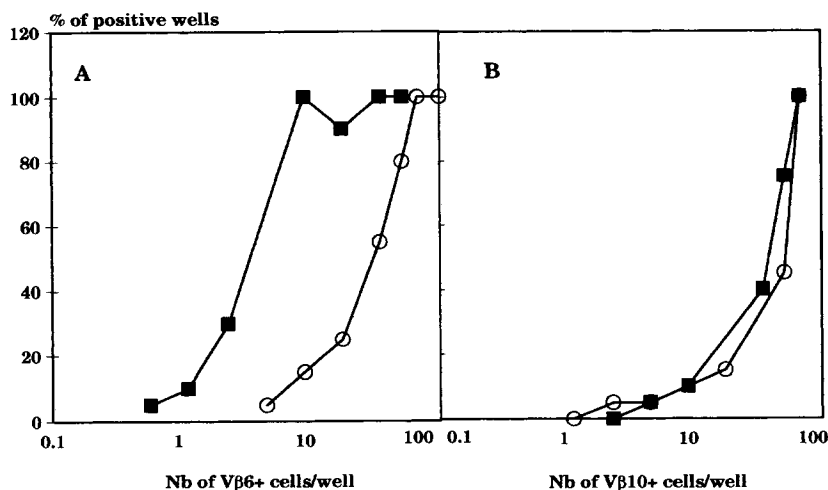
*Lymphocyte Responses to Influenza Virus Are Increased in SAg-treated BALB/c (Vβ6+), but Not in BALB/D2 (Vβ6-) Mice.* Having shown that rabies SAg treatment enhances the influenza response at the antibody level, we next addressed the question of whether rabies SAg affected the lymphocyte response. We compared the ability of lymphocytes from SAg + influenza- or influenza-injected mice to proliferate either without added stimulus (spontaneous proliferation) or with three different concentrations of influenza (influenza-specific proliferation). Two controls were included: mice receiving SAg or PBS alone. Draining popliteal LN were removed 1, 3, 5, and 7 d after the treatment, and LNC were treated with or without influenza. Results are presented for day 3 in Fig. 3. Without stimulus (0 μg/ml of influenza), LNC of SAg-treated BALB/c incorporated  $5 \times 10^3$  cpm more than LNC from mice receiving influenza alone. This difference was even stronger 7 d after the injection ( $3 \times 10^4$  cpm more than the [<sup>3</sup>H]thymidine incorporation of LNC from mice receiving influenza alone, data not shown). This indicates that spontaneous proliferation was enhanced by the SAg treatment.

Regarding the influenza-specific LNC proliferation, it can be seen that whatever the concentration of influenza (0.01–1 μg/ml), [<sup>3</sup>H]thymidine incorporation was five- to threefold higher in LNC from mice injected with SAg + influenza than from mice injected with influenza alone. This increase is evident as early as day 3 (Fig. 3), and maintained until 7 d after injection (data not shown). Taken together, the data clearly indicate that rabies SAg stimulates the cellular response, and suggest that rabies SAg could have enhanced the number of LNC recruited in vivo by the influenza virus.

In contrast, as shown in the right panel of Fig. 3, rabies SAg treatment did not amplify the [<sup>3</sup>H]thymidine incorporation of LNC from BALB/D2, indicating, as already stated for the antibody response, that the mechanism of the rabies SAg stimulating activity correlates with the presence of Vβ6 T cells. The absence of any increase in proliferation in BALB/D2 LNC raises two issues. First, since B cells of BALB/c and BALB/D2 are identical, the absence of a SAg stimulatory effect on BALB/D2 LNC strongly suggests that LNC proliferation did not result from direct B cell activation. Secondly, the possibility that the increase of [<sup>3</sup>H]thymidine incorporation in SAg-treated mice resulted from



**Figure 4.** In vivo rabies SAg treatment enhances IL-2 and IL-4 production by influenza-restimulated BALB/c LNC. In vitro IL-2 (left panels) and IL-4 (right panels) were compared in LNC cultures of BALB/c mice injected with SAg + influenza (■) and influenza (lines). LN were collected 3, 5, and 7 d after treatment. IL-2 was measured by CTLL-2 cell proliferation and IL-4 by capture ELISA. These data are representative of two separate experiments.



**Figure 5.** In vivo rabies SAg treatment enhances the number of influenza-primed T cells within the Vβ6+ population. LDA were performed with purified Vβ6 T cells (A) or Vβ10 T cells (B) isolated from LNC collected 7 d after injection, of mice receiving either SAg + influenza (■) or influenza alone (○). 20 replicate wells were seeded for each concentration of purified Vβ T cells, and 20 replicate wells without T cell as control. A well was considered positive when IL-2 production, detected in its supernatant, was at least three times higher than the average of controls. These data are representative of two separate experiments.

the administration of two antigens (SAg and influenza) is excluded.

*In Vivo Rabies SAg Treatment Enhances In Vitro IL-2 and IL-4 Production by Influenza Restimulated BALB/c LNC.* T lymphocytes are most probably responsible for the influenza specific proliferation of LNC observed above. To investigate this question and test whether in vivo SAg treatment enhances interleukin production, IL-2 and IL-4 were measured in the supernatants of LNC from BALB/c receiving SAg + influenza or influenza alone in vivo, and then cultured in vitro with increased concentrations of influenza. IL-2 production (Fig. 4, left panels) is shown for LNC collected on days 3, 5, and 7 after the injection of SAg + influenza or influenza alone. In response to influenza restimulation, IL-2 production is higher, occurs earlier and requires lower doses of antigen in LNC of SAg-treated mice than in LNC of mice injected with influenza alone.

**Table 1.** Influenza-primed T Cells Frequencies within Vβ Purified Populations

Injection	Purified population	Frequency ± SE	Confidence interval 95%
		$1/\text{cell Nb} \times 10^{-3}$	
a Influenza	Vβ6+	$1/41.8 \pm 0.2$	[20.7-79]
b Rabies SAg + influenza	id	$1/8.6 \pm 0.2$	[6.4-11.4]
c Influenza	Vβ10+	$1/70.1 \pm 6$	[41.9-112.3]
d Rabies SAg + influenza	id	$1/49.1 \pm 3.1$	[22.3-142.6]

Data described in Fig. 5 were regressed as the log of the percentage of nonresponder wells against the number of cells/well, and frequencies estimated as the reciprocal of the number of cell corresponding to 37% of nonresponders. Frequencies are presented ± SE and with their ranges (95% CI), calculated using the least square method of regression. Significances, assessed by variance analysis, were: (a/b)  $P = 0.004$ ; (a/c)  $P = 0.09$ ; (c/d)  $P = 0.6$ .

For example, when restimulated with 1 μg/ml of influenza antigen, LNC of SAg-treated mice produced enough IL-2 to induce a strong proliferation of CTLL-2 ( $10 \times 10^3$  cpm) whereas LNC of mice injected with influenza alone produced IL-2 levels that are undetectable by the CTLL-2 assay. 7 d after the in vivo injections, 1,000-fold more influenza antigen was necessary in vitro to induce detectable levels of IL-2 in LNC of mice primed with influenza alone compared to those primed with SAg + influenza. Similarly, but at lower levels, IL-4 production (Fig. 4, right panel) of LNC taken from SAg + influenza-treated mice was also significantly higher than that observed in LNC from mice receiving influenza alone. The kinetics of IL-4 influenza-specific production is, as expected, delayed in comparison with IL-2 production. Together these data clearly indicate that rabies SAg treatment results in a higher cytokine production in response to the antigen, and strongly suggest that mainly T lymphocytes account for the influenza-specific proliferation.

*Rabies SAg Specifically Increases the Number of Influenza-primed Vβ6 T Cells.* The next question addressed was whether rabies SAg specifically increases the number of influenza-primed Vβ6 T cells. To answer this, we quantified the number of influenza-primed T cells within the Vβ6 T cells population. This was measured by a LDA of purified Vβ6 T cells from LNC of BALB/c mice receiving SAg + influenza or influenza alone. As a control, frequencies of influenza-primed Vβ10 T cells, which are not recognized by the rabies SAg (6), were analyzed in the same conditions. The percentage of positive wells (out of 20) versus the number of cells ( $\times 10^{-3}$ ) per well are presented in Fig. 5. The estimated frequencies ± SE and the 95% confidence interval (Table 1) were then calculated for each group, as described in the legend. Within the Vβ6 T cell population, the frequency of influenza-primed T cells is nearly five times higher when mice have been injected with SAg + influenza ( $1/8.6 \times 10^3$ ) compared with mice injected with influenza alone ( $1/41.8 \times 10^3$ ). By comparison, within the Vβ10 T cell population the difference of frequencies is

much smaller ( $1/49.1 \times 10^3$  compared to  $1/70.1 \times 10^3$ ), and not significant. These results show that the rabies SAg, during establishment of the primary immune response, increases specifically the number of influenza-specific T cells bearing the recognized V $\beta$ 6 element.

## Discussion

In this study, we present evidence that rabies NC, through its SAg properties, clearly stimulates both at the T and B lymphocytes level, an associated, unrelated immune response. T cell proliferation and cytokine production are increased, as well as specific antibody secretion. This latter result is observed both during the primary and secondary response and, without modification of their characteristics such as isotypes or subclasses. We had found previously that rabies SAg targets the V $\beta$ 6-expressing CD4<sup>+</sup> T lymphocytes in mice (6). In the present study, we show that the adjuvant effect was not observed in congenic mice missing V $\beta$ 6 T lymphocytes, and moreover, that within the influenza responding T cells, it is primarily the V $\beta$ 6+ population which is increased. This indicates that V $\beta$ 6 T lymphocytes play a key role in the augmentation of the specific immune response and strongly supports our hypothesis that the SAg property of NC is responsible for its enhancing mechanism.

Liu et al. (14) described, at the clonal level, how SAg and conventional antigen trigger two distinct activation pathways. The signal provided by the SAg is described as acting in synergy with the antigenic one resulting in stronger proliferation and cytokines release. Applying this clonal study to our model, the coinjection of influenza antigen and rabies SAg, we can propose a cascade mechanism starting with the rabies SAg property to provide an additional activation signal to T cells. In this hypothesis, the first target of coinjected rabies SAg would be the influenza-specific T cells bearing a V $\beta$ 6 TCR, explaining the absence of effect observed in congenic V $\beta$ 6-negative mice. The extra-signal brought by rabies SAg on these cells would augment their frequency, as indeed observed in the LDA experiment. In a second step, the increased amount of cytokines released in the environment would favor the expansion of many influenza-specific T cells, irrespective of their V $\beta$  element, as reflected by the very strong enhancement of proliferation and cytokines production observed with the total LN cells. The overall increase in T cells number and released cytokines would then provide a greater help toward activated B cells and Ig production, as observed in the specific antibodies assays. Finally, the higher number of primed T and B lymphocytes might result in the enlargement of the number of memory cells, since we have observed that rabies SAg modulates the secondary response as well.

It is well documented that SAg can induce in vitro polyclonal B cell proliferation and differentiation, resulting in Ig production. This property has been described in vitro for nearly all viral, bacterial or mycoplasma SAg (15–17). However regarding their effect in vivo and on a specific immune response, two kinds of SAg can be distinguished. In

addition to the rabies SAg (this study and reference 8), the capacity to stimulate antibody production is also documented with the *Mycoplasma*-derived SAg (the *Mycoplasma arthritidis* mitogen, MAM), which was found to stimulate polyclonal IgM production and specific anti-OVA IgG (15, 18). However, other SAg are devoid of this capacity, in particular the bacterial SAg, the *Staphylococcus enterotoxin* type B, SEB, which inhibits in vivo antigen-specific antibody responses (19, 20). It seems then that SAg T-B bridging is not sufficient in vivo to trigger B cells to produce antigen-specific antibodies and that additional factors are involved. The nature of the cytokines produced upon SAg activation could be one of these factors, since they play a major role in modulating the capacity of lymphocytes to produce Ig. For example, it is known that high IL-2 and IFN- $\gamma$  production drives the immune response toward a cytotoxic response rather than an antibody response. Moreover, bacterial SAg, such as SEB (*Staphylococcus aureus* enterotoxin of type B) and TSST-1 (toxic shock syndrome toxin of type 1), were found to be strong IL-2 inducers (5, 21–23), whereas MAM and rabies SAg-reactive T cells secrete lower levels of IL-2 and no IFN- $\gamma$  (18). Another common feature between MAM and rabies SAg is that they are relatively weak T lymphocyte mitogens compared to bacterial SAg (15). Other arguments support the premise that these properties are involved in the capacity of SAg to enhance antibody production or not. First, addition of IL-2 to MAM-activated cultures diminishes drastically the ability of these cells to produce IgG (15). Second, the use of suboptimal doses (100-fold lower than those required for proliferation) of bacterial SAg restores their capacity to stimulate Ig production (24). In these experiments, the relationship between Ig induction and mitogenic effect is inversely proportional. Therefore, it is likely that bacterial SAg capacity to diminish antibody responses in vivo may be related to their high mitogen activity for T lymphocyte and strong IL-2 inducer capacity. Conversely, the ability of MAM and rabies SAg to stimulate an antigen-specific response in vivo may be related to the opposite properties: weak T lymphocyte mitogen and weak IL-2 inducer, compared to bacterial SAg.

Inhibition of antigen-specific antibody responses observed in vivo with most bacterial SAg could be due to another factor, the anergy or nonresponsiveness that most of them induce. It is well known that SEB, for example, exerts pleiotropic effects in vivo, causing first a rapid expansion of the V $\beta$  T cells recognized 36–92 h after injection, followed 4–5 d after injection by a clonal deletion and/or an anergy state of the previously expanded T lymphocytes (25). The SAg capacity to first activate and then anergize entire T lymphocyte subsets may explain the puzzling results obtained when varying the schedule of immunizations. For example, SEB given long enough after priming (at least 2 d) boosted the response or had no effect if given at the time of priming or before the antigen SEB suppressed the immune response (19, 20). Since NC is a SAg, it is surprising that the rabies SAg is a stimulant when given at the time of priming (or before), and, especially that it has

a long lasting effect. Anergy is defined as an absence of response of T cells to a second stimulation with the same antigen (26). We have shown here that the T cells primed by influenza and NC proliferated and produced more IL-2 to an in vitro recall of antigen. Similarly, in vivo, another study (8) showed that a recall of antigen (KLH) with NC in mice already primed with NC + KLH did not inhibit the antigenic response, but boosted it. These data confirm that rabies SAg, differing from most other SAGs, does not induce anergy in vivo. Anergy is thought to be induced when a stimulus is excessive, not associated with the right cosignals, and/or the stimulated cells are not mature enough. The absence of anergy induced by NC fits well with the unique properties of rabies SAg as a weak T lymphocyte mitogen, and activator of already differentiated B cells and primed T cells (5).

It therefore appears that, despite the capacity of every SAg to bridge together T and B cells, only a special class of SAg has adjuvant properties. These SAG must be weak T lymphocyte-mitogens, and low IL-2 inducers, and must not trigger anergy of the cells they expand. Currently, only two SAG fulfill these criteria: rabies NC and MAM. Bacterial SAG, in contrast, are suppressors of in vivo antigen-specific antibody production because of their excessive activation of the immune system.

Because of the unusual T-B interactions that rabies SAg may create, they can be suspected of reactivating T and B lymphocytes directed to auto-antigens. In a model of autoimmune disease where the recognition of the auto-antigen is restricted to a limited set of V $\beta$  T lymphocytes, it has been described that injection of SAG activating these crucial V $\beta$  T lymphocytes stimulates autoimmune relapse (27). Our results indicate that no increase of influenza antibodies could be obtained when NC was injected without antigen, showing that NC alone was unable to recall a memory response. It seems, therefore, that the risk linked to any potent immunostimulant of activating resting pathogenic T cells is minimal with rabies SAg. In this respect, it is possible that the relative weakness of NC is once again an advantage.

In conclusion, we propose that rabies SAg could be a good candidate as adjuvant in vaccine design because of its capacity to stimulate specific antibody production over a long period and its ability to enhance the establishment of a memory response. Moreover, except for HIV-infected patients (28), no V $\beta$  T lymphocyte deletion has been reported so far in humans, and SAG activation is not MHC restricted. It could thus be the first of a new class of T-mediated adjuvants.

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