Neonatal Deletion and Selective Expansion of Mouse T Cells by Exposure to Rabies Virus Nucleocapsid Superantigen

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Summary

The nucleocapsid (NC) of the rabies virus behaves as an exogenous superantigen (SAg) in humans. In the present report, we analyzed whether it is also a SAg in mice by studying the effect of NC on T cell receptor (TCR) Vβ expression in BALB/c mice. Repeated injection of NC in newborn BALB/c mice led to a marked reduction by two- to sixfold of Vβ6 expressing $CD4^+$ T cells in spleen and in peripheral blood. Decrease of Vβ6-expressing $CD3^+$ mature T cells was also observed in thymus. Single NC injection in footpad resulted in a three- to sixfold expansion of Vβ6 $CD4^+$ T cells, but not of $CD8^+$ T cells, in the draining lymph nodes of BALB/c mice. The intensity of the stimulation was dose dependent and was maximal 3 d after the NC injection. The clonal deletion of T cells bearing a particular Vβ demonstrates that NC is a SAg in mice. T cells, especially $CD4^+$ T cells, are an essential factor in host resistance to rabies virus and also in the pathophysiology of paralysis; thus, we postulate that a rabies virus component, which stimulates T cells, such as a SAg, may increase virus immunopathogenicity. To evaluate this hypothesis, we compared the course of rabies in adult BALB/c lacking Vβ6, 7, 8.1, and 9 T cells and in normal BALB/c. Immune-related paralysis was decreased in BALB/c missing the NC target VβT cells. Transfer of Vβ6 but not of Vβ8.1-3 T cells into recipient mice lacking Vβ6, 7, 8.1, and 9 allowed the immune-related paralysis to evolve. Taken together, these results strongly support the hypothesis that T cells expressing rabies SAg-specific Vβ6 T cells, are involved in the genesis of the immunopathology that is characteristic of paralytic rabies.

Superantigens (SAgs)[1] are defined by the capacity to stimulate a large fraction of T cells predominantly on the basis of the Vβ elements of the TCR (1). Unlike conventional antigens, they do not require antigen processing and bind outside the peptide binding groove by clamping the MHC class II and the TCR molecules (2, 3). The best studied SAgs are the bacterial enterotoxins produced by *Staphylococcus aureus*, the most potent T cell activators known so far (4). SAgs are also produced by other bacteria, mycoplasma (4, 5), and have been found to be encoded by exogenous and endogenous mouse mammary tumor retroviruses (MMTVs and Mtvs, respectively) (6–8). Retroviruses are known to be potentially severe immunopathological agents. This fact has spurred the search for new superantigens of viral origin in humans. Although SAg-like properties have been suggested for HIV-1 (3, 9) this remains controversial (10). To date, the only viral SAg described in humans is the rabies virus nucleocapsid (NC) and its major component, the N protein (11). NC consists of a viral strand of RNA covered with the three rabies viral core proteins, N, NS, and L. NC can bind to MHC class II molecules without processing and can stimulate in vitro Vβ8 human T lymphocytes (11).

When SAgs are encountered during T cell development, they induce a decrease of reactive T cells by clonal deletion or by anergy (12, 13, 14). Vβ-specific deletions occur in mouse pups due to the integration in the germ line of endogenous SAg encoded by Mtv provirus (15, 16) or due to infection by MMTVs present in the milk (17, 18). Injection of the exogenous SAg staphylococcal enterotoxin B (SEB) into newborn animals also leads to clonal deletion (19). However, before deletion, SAgs usually induce expansion of the target T cells bearing specific Vβ. This was the case for exogenous SAgs encoded by MMTV(SW) or for endogenous Mtv-8 and -9 SAgs (6, 20). Expansion or deletion of entire Vβ subsets by a SAg is likely to exert a dramatic effect on pathogenesis of infection and host defense mechanisms and therefore on the outcome of infection. It has been shown that clonal dele-

[1] *Abbreviations used in this paper:* LNC, lymph node cell; MMTV, mouse mammary tumor retrovirus; NC, nucleocapsid; SAg, superantigen; SEB, staphylococcal enterotoxin B.

tion of Vβ14 T cells obtained in transgenic mice by the overexpression of a Vβ14-specific MMTV, MMTV-C3H, prevents further MMTV-C3H infection (21). Similarly, deletion of Vβs induced by the presence of an endogenous Mtv in BALB/c has a drastic effect on resistance to experimental infection by the exogenous MMTV specific for the same target Vβ (22). Taken together, these results suggest that SAg-specific Vβs are crucial elements for the MMTV infection. As an explanation, it had been hypothesized that MMTV SAg amplifies MMTV infection because it stimulates the virus reservoirs, the B cells, with the help of the $CD4^+$ T cells (6, 23, 24).

Rabies virus infects almost all mammals including laboratory mice to which rabies virus strains have been adapted. T cells, especially $CD4^+$ T cells, are an essential factor in host resistance to rabies virus (25) and also in the appearance of paralysis which is thought to be an inopportune consequences of the immune response (26, 27). Since SAg stimulates T cells, it could be expected that a rabies SAg could activate the immune process and thus exacerbate the pathophysiological sequelae. An animal model is required to test this hypothesis.

In this work, we tested whether the NC SAg properties, already established in vitro with human T cells, existed also in mice. To do this, we analyzed whether NC can induce the expansion and the deletion of particular Vβs in BALB/c mice. Then, we studied the role of NC-specific Vβ T lymphocytes in the development of the clinical syndrome of immune-related paralysis.

Materials and Methods

Rabies NC. NC was purified from rabies virus–infected hamster (baby hamster kidney cells clone SR [BSR]) cell lysates through CsCl gradients as previously described (28). Cell cultures and virus seed lots were found to be mycoplasma free by specific hybridization, DNA staining and agarose isolation techniques.

Mice. Three types of mice were used in these experiments: (*a*) female BALB/c (H-2^d, I-E^+, Vβ3, 5, and 11 deleted) purchased from Janvier (St-Berthevin, France); (*b*) congenic BALB/D2 (H-2^d, I-E^+, Mls1a: Vβ6, 7, 8.1, and 9 deleted) (29), a gift from Martine Brulay-Rosset (Villejuif, France); and (*c*) female BALB/c infected with MMTV(SW) obtained from IFFA-Credo (l'Arbresle, France). These BALB/c mice are known to delete Vβ6, 7, and 8.1 as a consequence of infection by the milk-born MMTV, MMTV (SW) (18). During experiments, MMTV carriers and MMTV noninfected animals were housed in separated isolators.

Injections. Baby mice, born from BALB/c mothers free of MMTV(SW), were used for the neonatal deletion analysis. Newborn mice were inoculated i.p. each every other day after birth with 50 μg of NC for a 2-wk period following a protocol previously described (19). Control animals were injected with RPMI 1640 medium. To reduce variability, new-born mice from multiple litters were randomly assigned to either sham-injected or NC-injected litters. Mice were injected i.p. with 1 mg of hydrocortisone 2 d before sacrifice to eliminate immature thymocytes (30). Two mice in each litter were sacrificed 4, 5, 6, 9, and 12 wk after birth. These dates correspond respectively to 2–4, 6, and 10 wk after the last NC injection. Spleen, thymus, and blood were taken from each animal and double stained before being analyzed by cytofluorometry.

To analyze peripheral stimulations of T cells by NC, 8- to 11-wk-old female BALB/c, free of MMTV(SW), were injected with 3 to 80 μg of NC in each footpad according to an already described protocol (18). Control mice were injected with CFA by the same route. Popliteal lymph node cells (LNCs) were removed 3, 5, 9, and 12 d, disrupted and double stained for flow cytometry analysis.

Staining and Flow Cytometric Analysis. Splenocytes, thymocytes, and popliteal LNCs from both medium- and NC-injected animals were treated with buffered ammonium chloride to lyse erythrocytes and then washed twice in phosphate buffer saline containing Ca^{2+}/Mg^{2+} (PBS Ca^{2+}/Mg^{2+}). Circulating blood lymphocytes were obtained from blood of retro-orbital sinus, purified through Ficoll-Hypaque and washed twice in PBS Ca^{2+}/Mg^{2+}. Lymphocytes were incubated with one of the Vβ-specific biotinylated mAbs (Table 1). PE-conjugated streptavidin was used as a second step in conjunction with FITC-conjugated hamster anti-$CD3^+$ mAb 2C11 (31) or rat anti-$CD4^+$ GK1.5 (gift from M. Pierres, Centre d'Immunologie, Marseille Lumigny, France) or mouse anti-$CD8^+$ JR4.5 (gift from J. Rolland, Institut Pasteur, Paris, France). Cells were analyzed in a FACScan® cytofluorometer (Becton Dickinson & Co., Mountain View, CA). Mature thymocytes and blast LNCs were gated by forward and side-scatter analysis.

Infection of Mice with Rabies Virus. Mice were injected intramuscularly (i.m.) with 1×10^7 infectious particles of rabies virus Pasteur Virus strain (PV4) in both hind legs. Signs of weakness and paralysis and death were recorded every day up to 16 d after the virus injection. Weakness was determined by measuring the animal's ability to support its body weight with its hind legs. Paralysis was defined as a total loss of hind leg motility. In the rare cases of death, death by rabies was verified by checking for the presence of rabies virus in brains using immunofluorescence technique.

Transfer of Vβ T Lymphocytes. Vβ6 and Vβ8.1-3 T lymphocytes were extracted from spleens of 8-wk-old male BALB/c by panning using anti-Vβ6 and anti-Vβ8.1-3 mAbs (Table 1). 14-wk-old male BALB/D2 were transfused by tail vein injection of 14×10^6 Vβ6 or Vβ8.1-3 T splenocytes. Control mice were not transfused. Each group contained four animals. 1 h after cell transfer, mice were infected i.m. with rabies virus as described above.

Table 1. *Vβ-specific Monoclonal Antibodies*

Vβ	Name	Species	Reference
2	B20.6	Rat	39
3	KJ25	Hamster	40
4	KT4	Rat	41
5	MR9-4	Mouse	42
6	RR4-7	Rat	43
7	TR31O	Rat	44
8.1,2	KJ16	Rat	45
8.1,2,3	F23.1	Mouse, C57L/J	46
9	MR10-2	Mouse, SWR	47
10	B21.5	Rat	39
11	RR3-15	Rat	48
14	14.2	Rat	49
17	KJ23a	Mouse, SWR	1

Statistical Analysis. Differences between groups were analyzed using appropriate frequency analyses including student's *t*-test and chi-square calculations.

Results

Neonatal Deletion in Newborn Mice. To assess the SAg property of rabies NC in mice, we searched for clonal deletions of T cells expressing particular Vβ after neonatal injection. Percentages of T cells expressing Vβ2 to 11, and 14 in thymus and spleen of both control and NC-injected animals were estimated by cytofluorometry. In thymus, 6-wk after birth, the Vβ6 $CD3^+$ T cells from NC-injected mice showed a decrease compared to controls (Fig. 1, compare *B* and *A*). In spleen, 5 wk after birth, the number of $CD4^+$ T cells expressing Vβ6 drops almost completely in the NC-injected compared to normal mice (Fig. 1, compare *D* with *C*). Means of percentages of T cells expressing Vβ2 to 11 and 14 are shown in Fig. 2. In spleen, (Fig. 2 *A*), a significant drop of Vβ6 in NC-treated animals occurred (t = 5.48, degree of freedom [df] = 8, and p = 0.0006) whereas the drop in Vβ7 NC-treated $CD4^+$ T cells was not significant. In thymus, (Fig. 2 *B*) the percentage of Vβ6 was significantly reduced in NC-treated animals compared to sham-injected animals (t = 4.52, df = 5, p = 0.0062) whereas the drop of Vβ2 and Vβ7 $CD3^+$ thymocytes was not significantly altered in comparison with normal littermates. The percentages of the other Vβ were not modified, although the total number of mature thymocytes was slightly lower in NC-injected animals (60%) compared to controls (80%) (Fig. 1, compare *A* and *B*). In summary, these results demonstrated that neonatal injections of NC induce deletions of Vβ6 $CD4^+$ T cells in spleen and deletion of Vβ6 $CD3^+$ T cells in thymus.

Kinetics of Deletion in Newborn BALB/c Mice. The kinetics of the NC-specific Vβ6 deletion were studied in the thymus

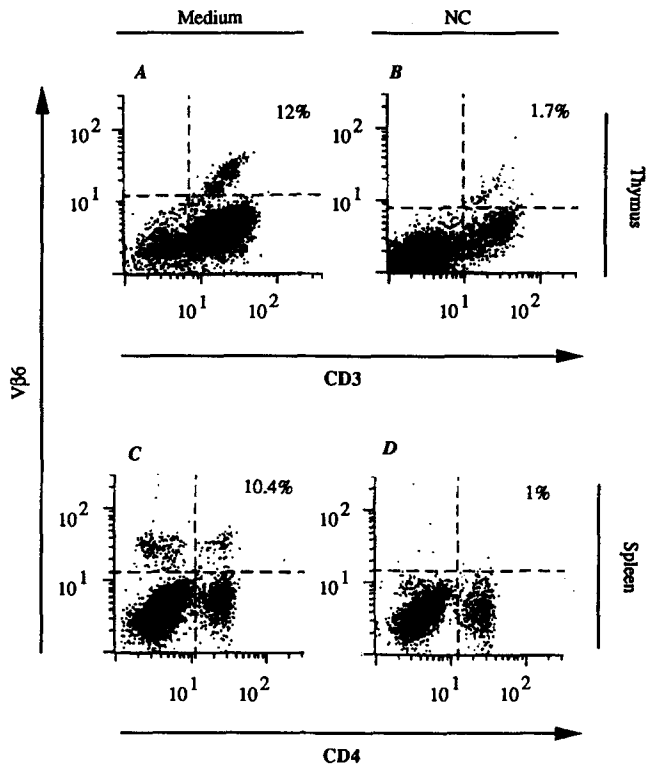

Figure 1. Cytofluorimetry analysis of Vβ6 T cells in spleen and thymus of NC-treated and nontreated newborn BALB/c mice. Thymocytes (*A* and *B*) and splenocytes (*C* and *D*) from both medium (*A* and *C*) and NC-injected (*B* and *D*) animals were double stained with biotinylated Vβ 6 specific mAb (Table 1) and in a second step, with PE-conjugated streptavidin and with either FITC-conjugated anti-CD4 mAb in the case of splenocytes or FITC-conjugated anti-CD3 mAb for thymocytes. Cells were analyzed in a FACScan® flow cytometer. Results are presented as log of fluorescence. The data depicted here are representative of all mice evaluated: the NC-injected mice had fewer Vβ6 than the noninjected mice.

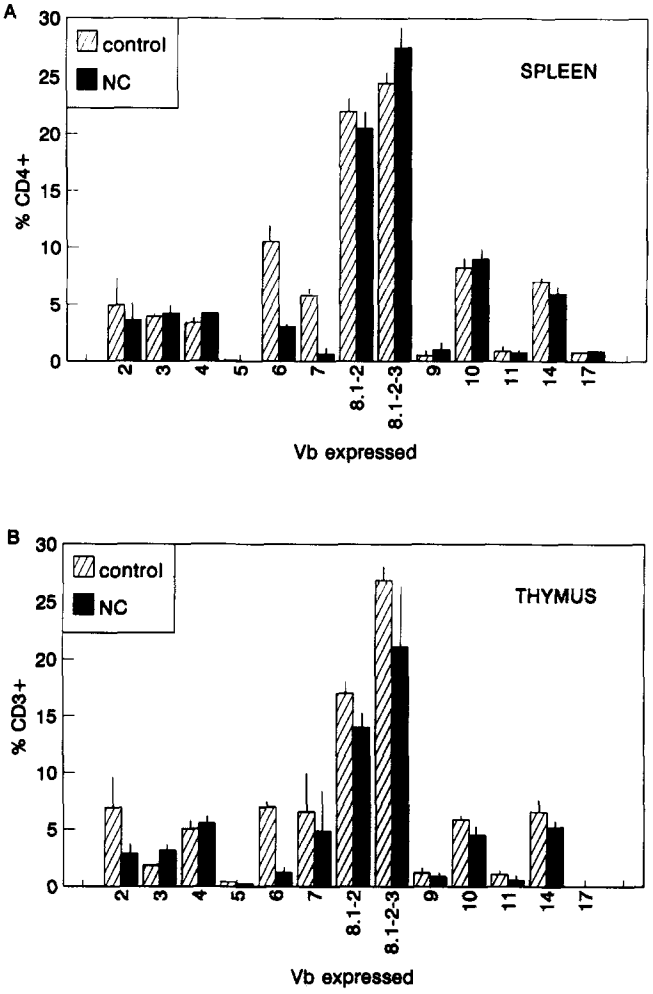

Figure 2. Neonatal deletion in spleen and thymus of BALB/c mice injected with NC. Analysis of TCR by (*A*) $CD4^+$ splenocytes and (*B*) $CD3^+$ thymocytes of NC-inoculated (■) and control 6-wk-old mice (▨). Splenocytes and thymocytes from both medium and NC-injected animals were double stained with one of the biotinylated Vβ-specific mAbs (Table 1) and then double stained and analyzed as in Fig. 1. Values are means of percentages +/− SD obtained in 4–2 mice of two separate experiments. Percentages of Vβ17 correspond to background of the double staining technique, since Vβ17 gene is not functional in BALB/c mice.

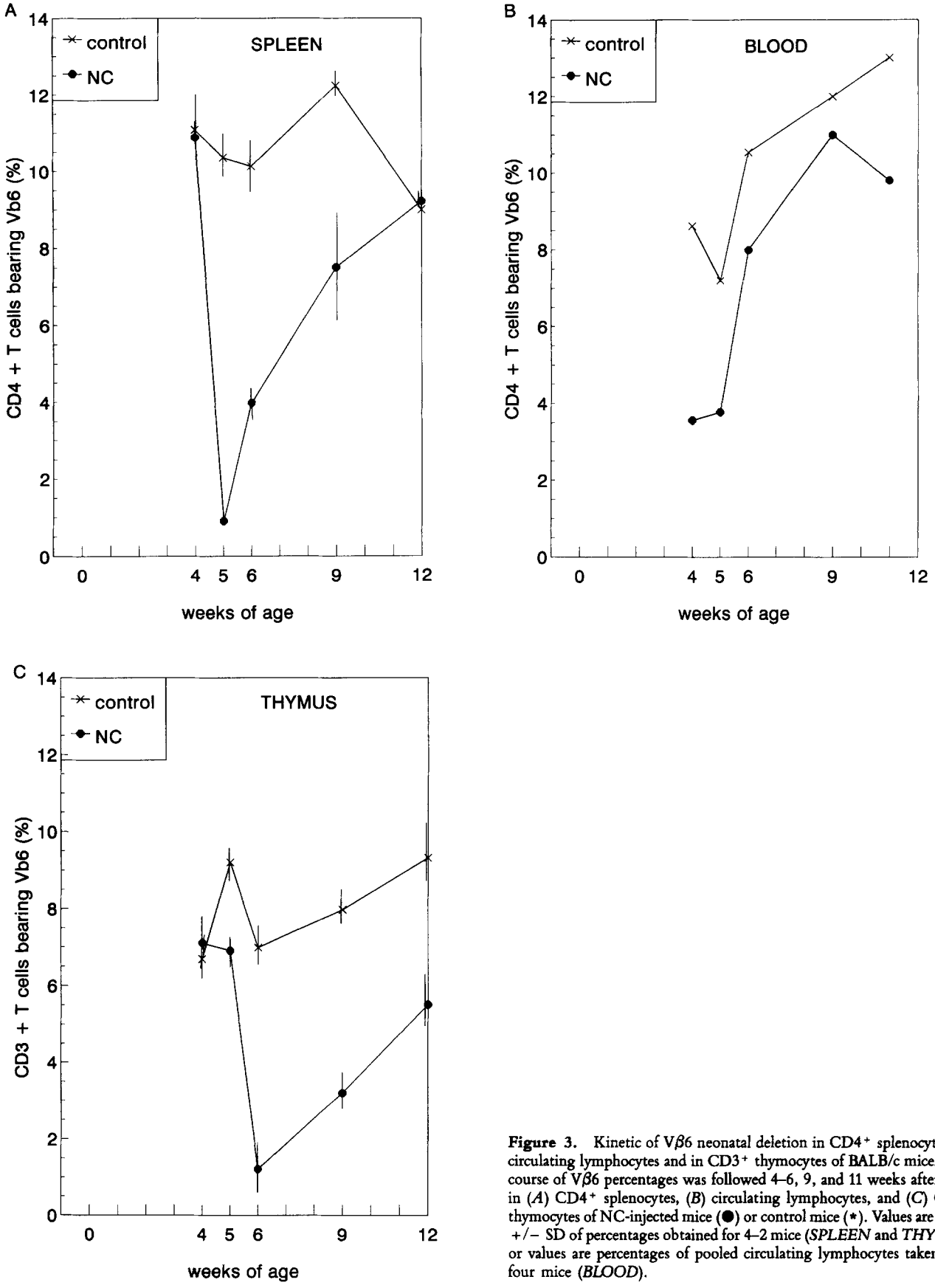

Figure 3. Kinetic of Vβ6 neonatal deletion in CD4+ splenocytes and circulating lymphocytes and in CD3+ thymocytes of BALB/c mice. Time course of Vβ6 percentages was followed 4–6, 9, and 11 weeks after birth in (*A*) CD4+ splenocytes, (*B*) circulating lymphocytes, and (*C*) CD3+ thymocytes of NC-injected mice (●) or control mice (*). Values are means +/− SD of percentages obtained for 4–2 mice (*SPLEEN* and *THYMUS*) or values are percentages of pooled circulating lymphocytes taken from four mice (*BLOOD*).

and in the spleen. Percentages of Vβ6 CD4+ T cells were determined as above in two NC-injected and two control litters at 4, 6, 9, and 12 wk after birth. In spleens, as shown in Fig. 3 *A*, deletion of Vβ6 CD4+ T cells was detectable and maximal 5 wk after birth. Percentage of Vβ6 CD4+ was still reduced in 6-wk-old mice but returned to control level (i.e., ~9%) thereafter. The time course of Vβ6 deletion was also studied in circulating CD4+ T cells taken from the blood of four NC-injected offspring (Fig. 3 *B*). Deletion was already achieved 4 wk after birth but disappeared after 6 wk. In thymus (Fig. 3 *C*), the time course study indicates that the Vβ6 CD3+ deletion was maximal at 6 wk. Return to normal Vβ6 levels seemed to be less rapid in thymus than in the periphery.

Selective Expansion of Vβ6 and Vβ7 Expressing T Cells in Adult BALB/c Mice by NC Inoculation. To analyze whether NC preferentially expands particular Vβs, we used the local injection technique which leads to a very strong T cell proliferation in the draining LNs. Adult BALB/c, 8–11-wk-old mice, free of exogenous MMTV(SW), were given footpad injections of NC, CFA, or medium alone. 3 d after NC injection, the popliteal LNs of NC-injected mice increased in size about fivefold in comparison with LNs of naive, medium-injected mice. This increase was also observed after CFA injection but not after injection of medium alone. Popliteal LNs from both legs of NC- or CFA-injected mice, were removed and the percentages of CD4+ LNCs bearing Vβ2, 4, 6–8, 10, or 14 were estimated by cytofluorometry. Analysis of the size and granulometry indicated that the LN population obtained from control animals is homogeneous and is exclusively composed of resting lymphocytes. In contrast, activation with NC, in the test group, or CFA, in the control group, was characterized by the appearance of a blast population. Percentages of Vβs obtained in CFA blasts were similar to those obtained in resting LNCs of naive mice (data not shown), indicating that the Vβ repertoire is not modulated by a non-specific inflammatory process. As shown in Fig. 4, the number of CD4+ blasts expressing Vβ6 increased significantly from 9.6 ± 1.7% in CFA-injected to 25.4 ± 0.9% in NC-treated LNCs ($t = 6.24$, $p < 0.0001$). The number of blasts expressing Vβ7 increased threefold from 4.6 ± 0.25% to 12.5 ± 0.6% ($t = 3.75$, $p = 0.0017$). In two separate experiments, NC injection led to an increase of Vβ6 of up to 62% of the CD4+ blasts in four animals. In these cases, percent of Vβ7 T cells was not modified, suggesting the existence of a compensatory mechanism during the expansion of particular Vβs (data not shown).

We demonstrated above that NC stimulates particular Vβ expressing CD4+ T cells. To test the effect of NC on CD8+ T cells, percentages of Vβ2, 6–8, and 14 were estimated among CD8+ LNCs. No significant changes of these Vβs could be observed between control and NC-injected LNCs (data not shown). This indicates that NC, in contrast to bacterial SAgs, does not stimulate peripheral mouse CD8+ T cells.

Kinetics of NC Vβ-specific Expansion and Dose–Response. Kinetics of expansion for Vβ6 and Vβ7 CD4+ LNCs were followed 3, 5, and 10 d after the local injection of NC. The

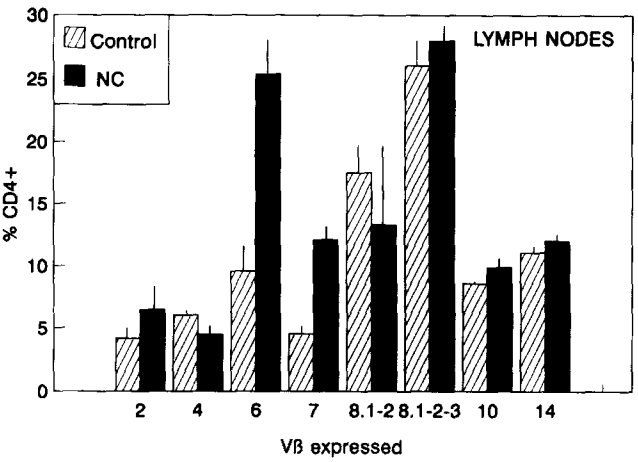

Figure 4. TCR Vβ repertoire of CD4+ T cells in popliteal LNs of adult mice 3 d after NC injection. LNCs from control and NC-injected 8–11-wk-old BALB/c mice were double stained and analyzed as described in Fig. 1. Blast CD4+ T cells were gated by forward and side-scatter analysis. Percentages given in black bars for eight NC-injected and hatched bars for three CFA-injected mice are the means +/− SD.

expansion of Vβ6 and Vβ7 CD4+ LNCs was transitory with a peak response 3 d after the injection (data not shown). The effect of different doses of NC was tested in BALB/c mice using increasing doses from 3 μg up to 80 μg per hind leg. It was found that injection of a minimal dose of 3 μg induced an increase of 11 to 17% in the Vβ6 CD4+ T cells 3 d after treatment. A maximal increase of 33% was obtained with 30 μg of NC, whereas injection of doses higher than 30 μg produced no further increases in the Vβ6 percentages: 29% with 50 μg and 25% with 80 μg.

Role of Vβ6 T Lymphocytes in Rabies Virus Immunopathology. Inasmuch as Vβ6 and possibly Vβ7 CD4+ T cells are specific targets of NC, we addressed the question whether these Vβs may interfere with rabies virus immunopathology by comparing the magnitude of immune-related disease in BALB/c mice expressing different levels of Vβ6 and Vβ7 T cells. Both exogenous MMTV, the MMTV(SW) and endogenous Mtv, the Mtv 7 or 44, cause Vβ6 and Vβ7 T cells deletion in infected BALB/c. In a first set of experiments, BALB/c infected by MMTV(SW) were used as a source of mice missing the NC-specific VβT cells. Percentages of mice showing signs of rabies-specific immunopathology, consisting of weakness and paralysis of the hind limbs, were recorded in MMTV(SW)-infected and in normal BALB/c. In both groups, hind leg weakness was detectable as early as 5 d after infection and paralysis appeared between day 7 and 10 and remained unchanged thereafter. At day 13, significantly fewer ($p < 0.05$) Vβ6-deficient mice than normal BALB/c showed signs of disease (Fig. 5, *top*). To avoid possible interference between MMTV and rabies infections, a similar experiment was performed in BALB/D2 mice bearing an integrated Mtv provirus, and thus missing constitutively Vβ6, 7, 8.1, and 9 T cells. As shown in the middle of Fig. 5, the mice lacking these Vβs were significantly ($p < 0.05$) more resistant to ra-

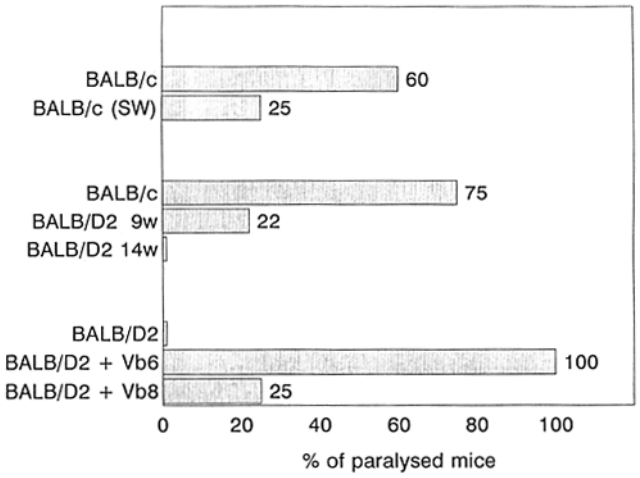

Figure 5. Impact of the percentages of Vβ6 T cells on the mouse susceptibility to rabies immunopathology. (*Top*) Percent of paralyzed mice bearing Vβ6 T lymphocytes (*BALB/c*) and in BALB/c mice missing Vβ6 as a consequence of MMTV(SW) infection (*BALB/c SW*). (*Middle*) Percent of paralyzed mice in BALB/c and in congenic BALB/D2 lacking Vβ6 because of the integration of the provirus Mtv-7. 9 and 14-wk-old BALB/D2 (*9w* and *14w*, respectively) were tested. (*Bottom*) Percent paralyzed mice in BALB/D2 transfused with either Vβ6 (BALB/D2 + Vb6) or Vβ8.1-3 (BALB/D2 + Vb8) splenocytes or nontransfused BALB/D2. Mice were infected in the hind limbs with rabies virus (1×10^7 PFU/mouse). Percentages of paralyzed mice were recorded 13 d after rabies virus injection. Each group of mice was composed of 12 mice with the exception of Vβ-reconstituted BALB/D2 groups which were composed of four.

bies paralysis (22 or 0%) than normal BALB/c (75%), indicating that absence of NC-specific Vβ T cells protect mice against immune disorders. Age is an important co-factor in resistance to rabies morbidity in Vβ6-deficient mice, since none of the 14-wk-old BALB/D2 developed signs of disease whereas 22% of 9-wk-old BALB/D2 became paralysed (Fig. 5, *middle*).

To demonstrate the role of rabies SAg-target Vβ T cells in rabies immunopathology, Vβ6 or Vβ8.1-3 T cells were transfused into BALB/D2 mice which normally lack these cells. Efficiency of Vβ6 reconstitution was checked 1 wk after transfer by analyzing circulating $CD3^+$ blood cells (Fig. 6). Range of Vβ6 $CD3^+$ T cells counts were as follows: Vβ6 transfused mice: 2.5–5.6% (Fig. 6 *E*) nontransfused BALB/D2 controls: 0.6–0.7% (Fig. 6 *A*) and BALB/c, control 9–11% (Fig. 6 *G*). In mice transfused with Vβ8 T cells, a few Vβ6 T cells, 1.7%, were detectable indicating that <1% (1.7–0.7%) of Vβ6 T cells were co-transferred with the Vβ8 T cells (Fig. 6 *C*). Reconstitution with Vβ8.1-3 T cells did not modify the percentage of circulating Vβ8 T cells, since the percent of Vβ8 T cells in Vβ8-reconstituted mice was similar, (24–27% in Fig. 6 *D*) to the Vβ8 percentage in Vβ6-reconstituted mice (25–28% in Fig. 6 *F*) or in the non-reconstituted mice (28% in Fig. 6 *B*). It is noteworthy that the anti-Vβ8 mAb, F23-1, which reacts with all three subsets of Vβ8 did not detect the well-documented decrease of Vβ8-1 in BALB/D2 (28% of Vβ8 are shown both in BALB/c and BALB/D2). This could be due to a better affinity of mAb

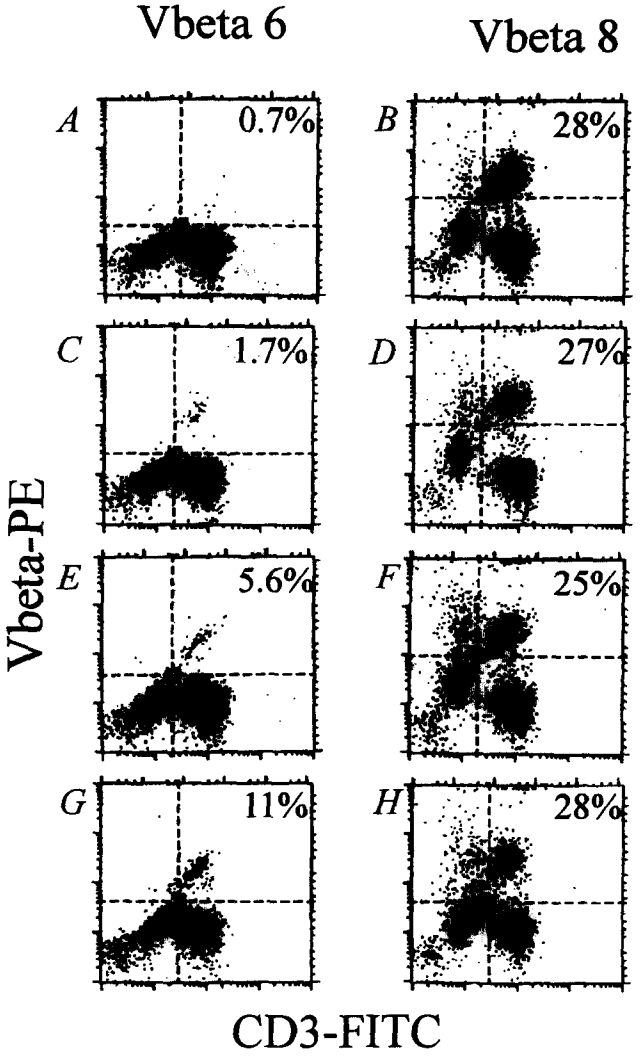

Figure 6. Efficiency of Vβ6 T cells transfer in BALB/D2 mice. Percentages of Vβ6 and Vβ8 T cells in circulating $CD3^+$ T cells were estimated by cytofluorometry as described in Fig. 1. (*A* and *B*) BALB/D2 mice missing Vβ6 and Vβ8.1; (*C* and *D*) BALB/D2 mice transfused with Vβ8(1-3) T cells; (*E* and *F*) BALB/D2 transfused with Vβ6 T cells, and (*G* and *H*) normal BALB/c. Results shown in this figure are representative data for mouse of each group.

F23-1 for the Vβ8.2 and Vβ8.3 than for the Vβ8.1 subset. Alternatively this may reflect compensatory mechanisms which keep constant the overall percentage of Vβ8 T cells. Rabies immunopathology was compared in the Vβ6, the Vβ8-transfused BALB/D2- and in nontransfused BALB/D2 (Fig. 5, *bottom*). All of the Vβ6-reconstituted BALB/D2 mice showed signs of severe paralysis as early as day 5, whereas all four of the nonreconstituted BALB/D2 mice remained free of paralysis. Among the Vβ8-reconstituted mice, only one, the one with 1.7% of Vβ6 T cells showed limb weakness which progressed to paralysis by day 10 (1/4:25%). Taken together these results demonstrate that rabies immunopathology is dependent on the presence of Vβ6 T cells.

Although the immunopathological sign of hind limb paralysis is common in these mice, death is rare. In contrast to what has been observed in BALB/D2 mice, where the PV4 rabies virus is rarely fatal (3 out of 35 infected mice), it is noteworthy that all the Vβ6 reconstituted BALB/D2 mice died of rabies by day 18.

Discussion

Recognition of a SAg by an host is a two-step reaction: first, SAg triggers the expansion of certain TCR Vβ subsets and later on, the expanded T cells enter into an unresponsive state and then die. An encounter at birth with SAg leads to a loss of most T cells expressing the reactive TCR Vβs during maturation in thymus (15). Clonal deletion of some entire Vβ subsets is regarded as a perquisite for a SAg. By showing that NC deletes T cells bearing particular Vβs in mice, we demonstrated that NC is a superantigen and we strengthened the previous results obtained in vitro with human lymphocytes where NC expands Vβ8 T cells, binds to surface class II molecules, probably by the α chain, and does not require processing (11). Taken together, these data lead to the conclusion that NC behaves as a SAg in both humans and mice and emphasize the point that viruses other than mouse retrovirus can also encode SAgs.

Demonstration that NC is a SAg for the mouse provides an experimental means to further investigate the role of rabies SAg in vaccination and in host infection. In this paper, we focused our attention on the role of rabies SAg in rabies infection and more precisely in rabies immunopathology. After rabies virus enters both sensory and motor nerve ending, it replicates in the neuronal cell bodies of the ganglia. Then, the virus invades the central nervous system. Rabies can result in two forms of disease: the encephalitic disease and the paralytic (32). In contrast to the encephalitic form of rabies, the paralytic form of rabies is characterized by a rapid clearance of rabies virus and by an almost complete absence of lethal outcome. Limb paralysis results from a peripheral immunopathological process which causes mononuclear cells infiltration and destroys the sciatic nerve (32, 26). Paralysis is not observed in immunosuppressed mice or in nude mice but is seen after T cell reconstitution, indicating that T cells play a crucial role in the induction of rabies paralysis (26, 32, 33). The mechanism of this process remains unknown. At least, it is not mediated by cytotoxic $CD8^+$ T cells, since removal of $CD8^+$ T cells does not protect against paralysis (27). We postulate that a component of the rabies virus which can stimulate T cells strongly, such as the NC SAg, may play a role in this process. Our data strongly support this hypothesis; we found that: (*a*) rabies immunopathology was decreased in BALB/c missing the NC-specific Vβs; (*b*) transfer of NC main targets Vβ6 T cells but not of Vβ8.1-3 T cells into deficient mice reactivated rabies virus immune-related pathology; and that (*c*) susceptibility to paralysis increases with the number of Vβ6 T cells. The finding that some of 9-wk-old BALB/D2 with 1.2% of Vβ6, were paralyzed whereas the 14-wk-old BALB/D2, with 0.6% of Vβ6) remain free of symptoms suggests that the 9-wk-old BALB/D2 show an immunopathological response to rabies because of the presence of a few remaining Vβ6 T cells (1.2%). In contrast the 14-wk-old BALB/D were protected because traces of Vβ6 (0.6%) were not sufficient to induce paralysis. Age dependence of the number of Vβ6 among BALB/D2 is consistent with the observation that complete deletion of Mtv targets Vβs is progressively acquired and only occurs after 10 wk (34). Similarly, Vβ8-transferred BALB/D2 mice expressing 1.7% of Vβ6 T cells were only partially paralyzed whereas all the mice expressing more than 2% of Vβ6 T cells were paralyzed. Thus, the threshold of Vβ6 T cells required for significant paralysis seems to be approximatively fixed around 2%. Altogether, these results strongly support the hypothesis that T cells expressing rabies SAg-specific Vβ6 T cells, are involved in rabies virus immunopathology.

We cannot rule out that other Vβ play a role in immunopathology since the mice which are less susceptible to rabies immunopathology also miss the Vβ7 T cells in addition to the Vβ6 T cells. These cells were also expanded in the periphery after NC injection (Fig. 4), however their expansion was less important than the Vβ6 T cells expansion. Moreover, the Vβ7 T cells were not significantly deleted after neonatal expansion (Fig. 2) suggesting that NC may have a better affinity for Vβ6 TCR than for Vβ7 TCR. The discrepancy between expansion and deletion of Vβ7 T cells by NC can be linked to the observation that expansion induced by superantigens, such as TSST-1, is not always followed by anergy and thus by deletion (35).

Previous observations that $CD8^+$ T cells are not involved in rabies immunopathology and the present finding that $CD8^+$ T cells are not specific targets of rabies SAg strongly support the hypothesis that SAg-related immunopathology is deserved only by the Vβ6 $CD4^+$ T cells.

Enhancement of rabies immunopathology by NC may be obtained because NC triggers an efficient Vβ6 $CD4^+$ T cell–mediated immune response which destroys the infected neurons in the periphery. Neurons normally do not express MHC class II molecules. INF-γ is able to stimulate MHC class II production. It cannot be excluded that SAgs which are known to make T cells to release large amounts of cytokines (36, 37), could trigger MHC class II expression on neuronal cells which became suitable targets for cytotoxic T cells. Alternatively, destruction of neurons could be obtained indirectly by killer microglia cells with the help of antibodies, via the mechanism of antibody-dependent cell cytotoxicity, the ADCC. Evidence has been obtained in mice that NC stimulates the production of neutralizing antibodies directed against the rabies virus envelope protein (38) suggesting that rabies SAg can induce cognate T-B interactions. In this hypothesis, rabies SAg could enhance the antibody response and induce an antibody-related immunopathology. It is not yet clear whether this immune response is sufficient to rid the nervous system of rabies virus. In the case of rabies, the balance between the immune response and the virus infection seems to play a key role in the issue of the disease. Several reports have noted that infected individuals died of rabies despite a strong immune

response suggesting that, most of the time, the immune response is launched too late to be efficient. Delay in the establishment of the immune response could explain the discrepancy observed between the issue of the transfer experiment, where BALB/D2 + Vβ6 died, and the experiment where NC induces protection (38). In our reconstitution experiment, Vβ6-transfused mice died despite a strong paralysis, whereas after the injection of NC a few hours or days before virus, paralyzed mice were protected (38).

The finding that NC can exacerbate the rabies immunopathology in mice raises the questions about a possible role for NC in producing the high incidence of neurological complications that follows vaccination in humans using rabies vaccines prepared in animal brain. Rabies infection can be prevented in humans exposed to rabid animals by prompt administration of rabies vaccine. In several countries, such as Brazil or India, economic reasons necessitate the use of rabies vaccines prepared from inactivated rabies-infected animal brains despite the neurological complications. Multiple injections of this vaccine over a 2-wk period are required to trigger an efficient immune response. We found that, in contrast to rabies vaccine prepared in tissue culture, the animal brain vaccine contains large amounts of NC (Montano-Hirose, J. A., and M. Lafon, manuscript in preparation).

More experiments are needed to establish whether neuropathological vaccine disorders may be linked to the presence of NC in brain rabies vaccines and to understand the mechanisms that can allow protection to rabies infection. The answers to these questions, in addition giving new insights in the role of SAg in infection, will have considerable consequence for vaccine design.

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