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Immunology

Rabies ribonucleocapsid as an oral immunogen and immunological enhancer

D. CRAIG HOOPER, ISABELLE PIERARD, ANNA MODELSKA, LASZLO OTVOS, JR., ZHEN FANG FU, HILARY KOPROWSKI, AND BERNHARD DIETZSCHOLD

The Center for Neurovirology, Department of Microbiology and Immunology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107-6799

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ABSTRACT **The administration of rabies ribonucleocapsid (RNP) by oral as well as parenteral routes was found to prime specific T cells and elicit N-protein-specific antibodies. *per os* and intramuscular immunization led to the production of antibodies of the IgA and IgG isotypes, respectively. Mice primed orally with RNP produced significantly enhanced amounts of virus-neutralizing antibody, compared with nonimmune controls, upon subsequent parenteral booster immunization with inactivated rabies virus. Thus oral immunization with rabies RNP primed cells capable of mediating a secondary systemic response to rabies virus. The results of experiments in which peptide and protein antigens were administered either physically coupled to or mixed with RNP indicate that RNP has an inherent capacity to enhance immune responses.**

While oral immunization has generally proven to be problematical, both live-attenuated rabies and vaccinia-rabies glycoprotein recombinant virus delivered in edible bait have proven to be effective vaccines (1–3). Since rabies virus infection initiates from skin or muscle by a bite, it is clear that the ingested bait vaccines induce a systemic immune response rather than the mucosal response and systemic tolerance that often accompany the feeding of antigen (4–6). It is conceivable that the ability of the live vaccines to replicate is important in generating systemic immunity when administered *per os* (p.o.). However, there is recent evidence to suggest that ingested killed rabies virus or rabies virus proteins also induce protective immunity (7, 8).

Although virus-neutralizing antibodies are invariably directed at the rabies glycoprotein, several previous studies have indicated that protective immunity to infection with rabies virus can be engendered by the intramuscular (i.m.) administration of the rabies virus ribonucleocapsid (RNP) (9). Antigenic determinants borne by the N protein of RNP have been implicated in this immunoprotective effect (10), which is, at least at its inception, independent of virus-neutralizing antibodies. Since the antigenic composition of rabies nucleoprotein is more highly conserved than that of the glycoprotein (11), a nonreplicating vaccine based on RNP may have more universal applicability. In this investigation we have therefore assessed the immunogenicity of orally administered rabies RNP.

METHODS

Viruses and Antigens. The fixed rabies strains Evelyn–Rokitnicki–Abelseth (ERA) and challenge virus standard (CVS-11) were propagated on BHK-21 cell monolayers and purified as described (12). The purified virus was suspended in phosphate-buffered saline (PBS), inactivated with β-propiolactone (βBL), and adjusted to a protein concentration of 100 μg/ml. Viral RNP was isolated from rabies virus-infected BHK-21 cells (13), and rabies N protein was purified from insect cells infected with a recombinant baculovirus expressing rabies N protein as described (14). Keyhole limpet hemocyanin (KLH; catalog no. H-2133) as well as staphylococcal enterotoxins A (SEA; S-9399) and B (SEA; S-4881) were purchased from Sigma. CLTB-36, a human immunodeficiency virus 1 (HIV-1)-derived synthetic peptide including a T-helper cell epitope from the C-terminal end of the core protein p24 and a B-cell epitope from the principal neutralization determinant of V3(MN), as well as HIV-1 pseudovirus were supplied by Connaught Laboratories. CLTB-36 was chemically synthesized using an Applied Biosystems model 430A peptide synthesizer and was coupled to RNP (RNP-CLTB conjugate) as described (15).

Determination of Antibody Titers. The neutralizing activity of serum from mice immunized with CVS-11 virus was determined as described (16). Anti-RNP antibody titers were determined by the indirect fluorescent antibody staining technique detailed in ref. 11. Radioimmunoassay (RIA) for N-protein-specific antibody as well as enzyme-linked immunoabsorbent assay (ELISA) for KLH and HIV-specific antibody were performed by conventional means using polystyrene plates (Dynatech; Immunolon 4) coated with baculovirus-expressed rabies N protein, KLH, and HIV pseudovirions, respectively. Following incubation with test sera, antibody specifically bound to the plates was detected using specific anti-mouse immunoglobulin reagents. For RIA, ^{125}I-labeled rabbit anti-mouse IgG (heavy chain-specific, mixed γ1, γ2a, and γ2b) or rabbit anti-mouse IgA (α heavy chain-specific) was used. Peroxidase-conjugated goat anti-mouse IgG (whole molecule, Sigma no. A-4416) was employed in an ELISA with 3,3′,5,5′-tetramethylbenzidine dihydrochloride as substrate. The color change following the addition of H_2SO_4 to stop the reaction after 30 min was measured at 450 nm in a Bio-kinetics reader (Bio-Tek, Winooski, VT).

Mice and Immunization. The C3H/HeJ and BALB/cByJ mice used throughout this investigation were 6- to 8-week-old female animals obtained from The Jackson Laboratory. To prime for rabies virus-neutralizing antibodies, 6-week-old C3H mice were immunized intraperitoneally (i.p.), i.m., or intradermally (i.d.) with 0.4, 2, and 10 μg of RNP or p.o. with 50 μg of RNP with and without 10 μg of cholera toxin B subunit. Throughout the investigation, p.o. immunization was carried out by placing 50 μl of antigen diluted in PBS in the oral cavity. Two weeks after priming, groups of 10 mice primed with either RNP or PBS were challenged i.p. with 5 μg of inactivated ERA virus (ERA-βPL). Blood was collected 10, 45, and 158 days after booster immunization. For the stimulation of rabies RNP-specific T cells, 6-week-old

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Abbreviations: CVS, challenge virus standard; ERA, Evelyn–Rokitnicki–Abelseth; i.d., intradermal; i.m., intramuscular; i.p., intraperitoneal; KLH, keyhole limpet hemocyanin; p.o., *per os*; RNP, ribonucleocapsid complex; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; HIV, human immunodeficiency virus; βPL, β-propiolactone; APC, antigen-presenting cell.

female C3H mice were immunized with 5 μg of RNP i.m. or 20 μg of RNP p.o., either a single time or twice with an interval of 10 days. The RNP-CLTB conjugate, either 5 μg i.m. or 20 μg p.o., was administered twice with an interval of ≈10 days to female C3H and BALB mice. To assess the capacity of coupled versus mixed RNP and CLTB to prime T cells, female C3H mice received a single i.m. dose of either 5 μg of CLTB or RNP alone, 5 μg each of CLTB and RNP in a mix, or 5 μg of RNP-CLTB conjugate. In all cases, the mice were sacrificed and antigen-specific splenic T-cell proliferation was assessed at least 10 days after immunization.

To assess the effect of RNP on the immune response to heterologous antigens, KLH alone, RNP, or a mix of RNP with KLH was administered i.m. (5 μg of RNP with and without 20 μg of KLH, 20 μg of KLH alone) to groups of mice twice with an interval of ≈10 days. Serum was obtained both prior to and 10–14 days following the second immunization. Antigen-specific proliferation of splenic T cells was determined coincident with the second bleed.

To prime for the production of HIV pseudovirus-specific antibody, 8-week-old male BALB mice received 20 μg of RNP-CLTB conjugate in PBS p.o. twice at an interval of ≈3 weeks and were challenged with HIV pseudovirus 20 days afterward. Serum was obtained 2 weeks later and HIV-specific antibody titers were assessed in ELISA. Control mice received only the pseudovirus.

Cell Culture and Antigen-Specific Proliferative Assay. Cells were prepared and cultured as described in detail elsewhere (17). Briefly, single cell suspensions were prepared from aseptically removed spleens by teasing through stainless steel mesh in PBS. Red cells were lysed by hypotonic shock and T cells were isolated by panning on goat anti-mouse immunoglobulin-coated plates. T cells (250,000) from various groups of mice were cultured with unselected spleen cells (100,000–250,000) from nonimmune mice as a source of antigen-presenting cells (APCs) in 200-μl volumes in round-bottom microtiter plates (Falcon) in the presence of the antigens indicated in the figure legends. Alternatively, T cells were cultured at 0.8×10^6 per ml with 1×10^6 irradiated (1000 rads from a cesium source; 1 rad = 0.01 Gy) spleen cells per ml in 2 ml in 24-well flat-bottom plates (Falcon). The medium employed was the α modification of minimum essential medium (GIBCO) supplemented with 4 mM L-glutamine (GIBCO), 0.05 mM 2-mercaptoethanol (Sigma), 25 mM Hepes, 10 μg of gentamycin/ml, and 0.5–0.6% fresh mouse serum. At the indicated times, microtiter cultures or 100-μl samples from 2-ml cultures were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine (specific activity, 65 Ci/mmol; 1 Ci = 37 GBq; ICN) for 4 hr. The cultures were then harvested using a multiple sample harvester (Skatron, Sterling, VA) and the water-insoluble radioactivity was counted on an LKB rack β-counter (LKB–Wallac) using conventional liquid scintillation techniques.

RESULTS

T-Cell Response to Rabies Virus Following Administration of RNP. Fig. 1*A* shows the results of an experiment designed to examine whether oral immunization with RNP elicits systemic proliferative T cells capable of responding to rabies virus *in vitro*. RNP was administered either i.m. or p.o. in a single dose or in two doses with a 10-day interval. T cells isolated from the spleens of immunized mice proliferated *in vitro* in the presence of rabies virus regardless of the route of immunization, indicating that T cells had been primed in each case. However, the proliferative response of T cells from orally primed animals was somewhat lower and developed more slowly than that of mice immunized i.m. Moreover, the T-cell response to RNP was significantly enhanced when

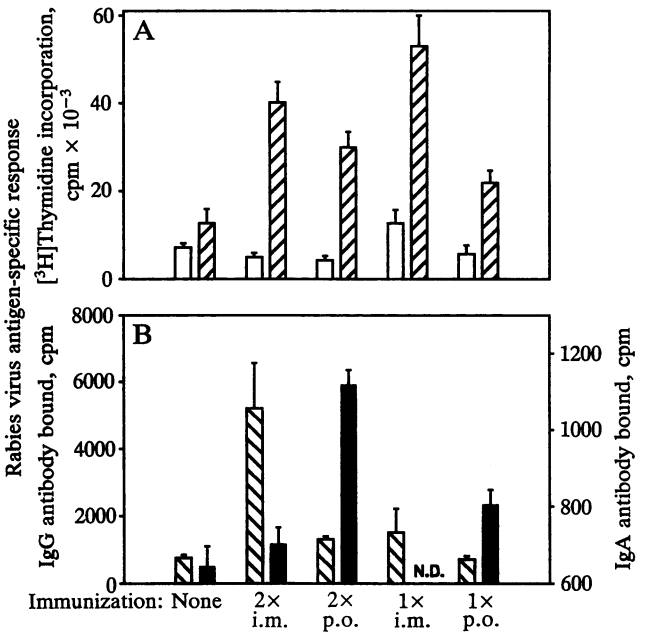

FIG. 1. T-cell proliferative response to rabies virus (*A*) and serum antibody response to rabies N protein (*B*) following immunization with RNP by different routes. Mice (C3H) received either a single dose (10 days before assay) or two doses (10 and 20 days before assay) of RNP i.m. (5 μg per dose) or p.o. (50 μg per dose). T-cell proliferation was determined as described in the text. (*A*) Peak proliferative response in the absence of added antigen (□) and in the presence of 10 μg of UV-inactivated rabies virus per ml (▨), which occurred after 96 hr of incubation. The proliferation of nonimmune cells at 96 hr of culture is also shown. Serum samples were obtained from the mice described above and assayed in solid-phase RIA for the presence of antibodies specific for rabies virus N protein. (*B*) N-specific IgG (▨) and IgA (■), displayed as mean cpm ± SD of the ^{125}I-labeled anti-isotype specific antibody bound. ND, not determined.

p.o., but not i.m., immunized mice were given a second dose of antigen by the same route.

The proliferative T cells raised by administration of RNP p.o. and by other routes respond *in vitro* to rabies virus, purified RNP, as well as purified N protein (data not shown). This is in agreement with previous studies indicating that rabies virus N protein expresses determinants stimulatory for T cells (10). However, while oral administration of RNP repeatedly resulted in an elevated splenic T-cell response to rabies virus, RNP, and N protein, oral administration of N protein failed to have any comparable effect (data not shown).

Oral RNP Immunization Elicits Serum IgA Antibody. Serum samples from the mice whose T-cell responses were assessed in Fig. 1*A* were analyzed in a RIA for anti-rabies N-protein antibody. The results of these tests, shown in Fig. 1*B*, suggest that there is no systemic IgG antibody response to N protein following p.o. administration of the complex despite stimulation of the systemic T-cell compartment. However, a low but significant titer of IgA anti-N antibody was detected in the serum of mice immunized twice p.o. This contrasts with the significant titers of anti-N IgG, but not N-specific IgA, found in mice that received two doses of RNP i.m.

Enhanced Neutralizing Antibody Response by Priming with RNP. To test the assumption that primed RNP-specific T cells detected systemically following p.o. administration of RNP could possibly have a role in immunity to rabies virus, several doses of RNP were administered to different groups of mice by various routes and the animals were subsequently immunized i.p. with inactivated rabies virus (ERA-βPL). In an

attempt to enhance the p.o. immunogenicity of RNP, one group of mice received a mixture of RNP and cholera toxin B subunit, which has been demonstrated to facilitate mucosal immunization (18, 19). Serum virus-neutralizing antibody titers, which have been correlated with protection from infection, were then determined at various intervals and these are shown in Fig. 2*A*. Fig. 2*B* shows a summary of the results obtained with optimal doses of RNP given by the different routes. Regardless of the route of administration, priming with RNP significantly enhanced the antiviral antibody response.

Capacity of RNP To Function as a Carrier for Peptide Antigens. To further examine the carrier effect of RNP evident in the experiment detailed above, we coupled RNP to a peptide antigen, CLTB-36. To ensure that the HIV-derived peptide CLTB-36 conjugated to RNP retained its antigenicity and determine whether it might be immunogenic when administered p.o., we immunized BALB/c mice p.o., as well as C3H mice p.o. and i.m., and tested splenic T cells from these animals for the capacity to respond to CLTB-36. As can be seen in Fig. 3, a strong proliferative response to CLTB-36 was obtained with cells from C3H mice immunized i.m. (Fig. 3*C*), while little reactivity was shown by cells from nonimmune C3H mice (Fig. 3*D*). Primed CLTB-36-specific T cells could be detected in BALB (Fig. 3*A*) and, to a lesser extent, C3H mice (Fig. 3*B*) immunized p.o. As shown in Fig. 4, the ability of BALB mice to produce anti-HIV antibodies following i.p. immunization with different doses of the pseudovirus is significantly enhanced by prior feeding of RNP-CLTB conjugate.

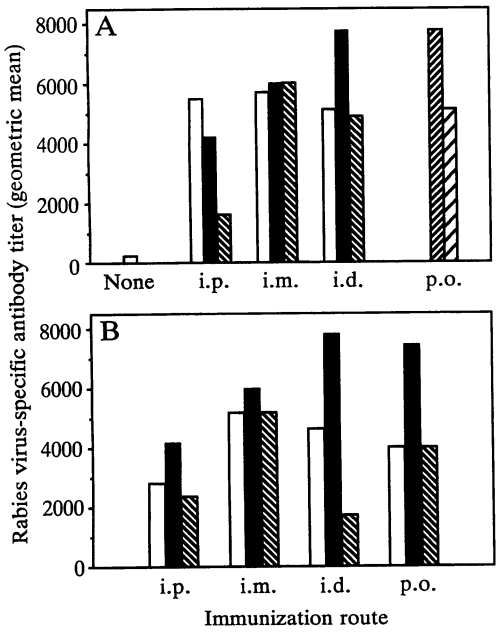

FIG. 2. Dose–response (*A*) and time course (*B*) of anti-rabies virus antibody following priming with RNP and challenge with rabies virus. Groups of 10 mice were either left untreated or immunized by different routes with various doses of RNP and, in the case of oral immunization, with RNP mixed with cholera toxin B. Ten days later the mice were challenged with ERA-βPL. The mice were bled at different time intervals, and serum anti-rabies virus titers were determined by immunofluorescence. (*A*) Geometric mean serum anti-rabies virus antibody titer of each group for the second bleed (45 days postchallenge). The RNP doses employed for priming in *A* were 0.4 (□), 2 (■), and 10 (▨) μg or 50 μg without (▨) and with (▨) 10 μg of cholera toxin B. (*B*) Serum titers of the groups of mice found to be optimally immunized by the different routes (2 μg for i.p., i.m., and i.d. immunization; 50 μg for p.o.) at first (□, 10 days postchallenge), second (■, 45 days), and third (▨, 158 days) bleeds.

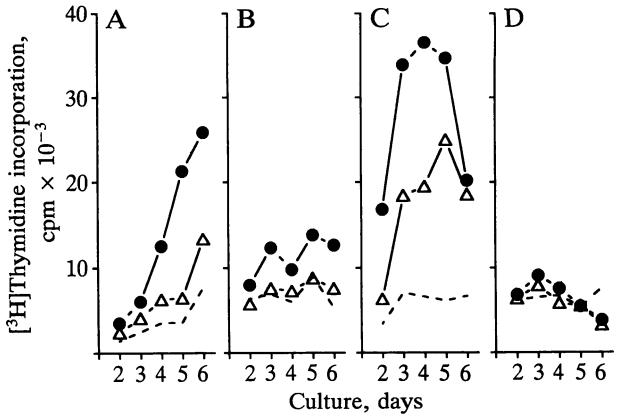

FIG. 3. T-cell proliferative response to CLTB-36 following i.m. and p.o. administration of RNP-CLTB conjugate. BALB/cByJ (*A*) and C3H/FeJ (*B–D*) mice either were left untreated (*D*) or received two doses of RNP-CLTB p.o. (20 μg; *A* and *B*) or i.m. (5 μg; *C*) ≈10 and 20 days before assay. The [3H]thymidine incorporation of cells cultured without added antigen (- - -) or in the presence of 1 (△) or 10 (●) μg of CLTB-36 per ml is shown.

Mechanism of Enhancement and Potential Adjuvant Effect of RNP. The results of the experiments presented above are consistent with the concept that rabies RNP is a particularly good antigen that maintains its immunogenicity when administered p.o. It is conceivable that the known resistance of the RNP complex in acid conditions is responsible for its capacity to immunize when given p.o. Alternatively, or in addition to its stability, RNP may have some inherent antigen-specific or nonspecific capacity to stimulate immunity. For instance, a previous investigation has concluded that rabies virus nucleocapsid may be a superantigen for human T cells (20). Since superantigens such as SEA and SEB act on both human and murine cells (21), it is conceivable that this property could contribute to the immunogenicity of RNP in our experiments. We therefore assessed, in our system, the ability of RNP to stimulate T cells and APCs from the spleens of nonimmune C3H mice by comparison with SEA and SEB (Fig. 5). It is clear that the rapid stimulatory effects of SEA and SEB, which can be seen in the first 24–48 hr of culture, are not shared by RNP.

To examine other possibilities for the strong immunogenicity of rabies RNP, we used i.m. rather than p.o. administration to minimize any contributions from the acid stability

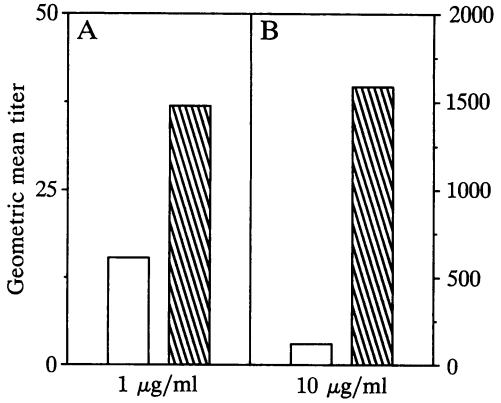

FIG. 4. Enhanced antibody response to HIV pseudovirus following p.o. administration of RNP-CLTB conjugate. As described more fully in the text, mice either were left untreated (□) or received 20 μg of RNP-CLTB p.o. twice (▨) and were then immunized i.p. with either 1 μg (*A*) or 10 μg (*B*) of HIV pseudovirus per ml. Serum anti-HIV pseudovirus titers were determined 2 weeks later by ELISA.

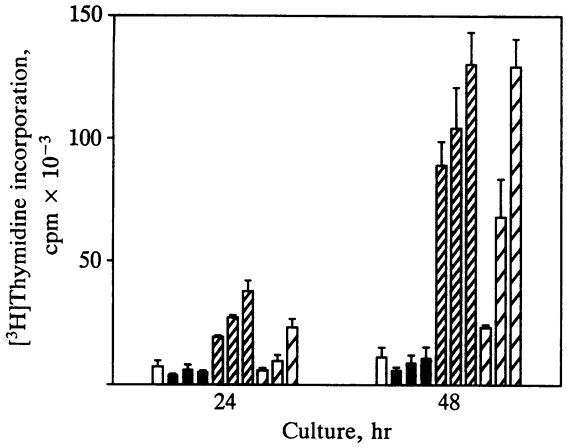

FIG. 5. Comparison of the stimulation of nonimmune T cells and APCs by RNP, SEA, and SEB. Isolated T cells plus an equal number of unselected spleen cells from nonimmune C3H mice were cultured in microtiter wells as described in the text in the absence of antigen (□) and in the presence of RNP (■), SEA (□), or SEB (□) at 10, 1, or 0.1 μg/ml. The results (mean ± SD) of the responses to increasing concentrations of antigen are depicted left to right.

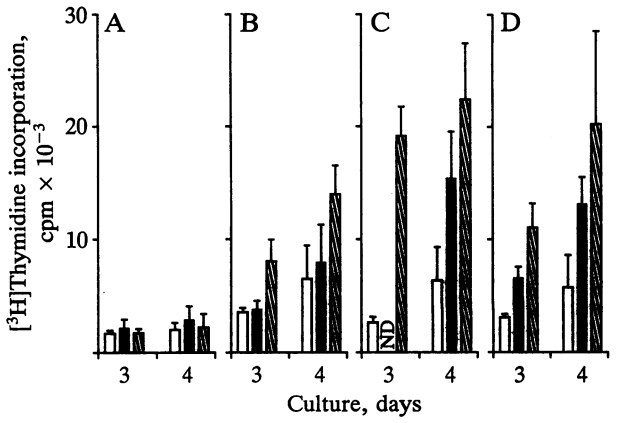

FIG. 6. T-cell proliferative response to CLTB-36 *in vitro* following i.m. administration of RNP and CLTB-36 separately, mixed, and as a conjugate. Mice received a single i.m. dose of either 5 μg of RNP (*A*), 5 μg of CLTB-36 (*B*), 5 μg of RNP-CLTB conjugate (*C*), or a mix of 5 μg of RNP plus 5 μg of CLTB-36 (*D*) 10 days before assay. Spleen T cells plus APCs were cultured without added antigen (□) and with 1 μg (■) and 10 μg (□) of CLTB-36. The peak proliferation (mean cpm ± SD) in the cultures, which occurred at either 3 or 4 days of incubation, is shown.

of RNP. To distinguish between antigen specific and nonspecific effects, we next compared the immunogenicity of RNP–peptide conjugate with that of a mix of the two components. Fig. 6 shows that i.m. immunization of mice with CLTB alone elicits only a weak response to a high *in vitro* concentration of CLTB-36. Enhanced responses to both high and low concentrations of CLTB-36 were obtained when either RNP-CLTB conjugate (Fig. 6*C*) or a mix of RNP plus CLTB (Fig. 6*D*) were used to immunize.

Further experiments have demonstrated that RNP can also enhance humoral immune responses to more complex antigens such as KLH. Fig. 7 shows the results of a comparison of i.m. immunization with KLH alone versus a mix of KLH plus RNP. A single dose of a mix of KLH plus RNP was sufficient to induce strong antibody responses to KLH in four of the five mice tested, which were significantly greater than the serum anti-KLH titers seen following administration of KLH alone (data not shown). A second immunization with the same reagents boosted the anti-KLH response in both KLH alone and KLH plus RNP groups, although a large difference in the mean titers was maintained (Fig. 7*A*). In this case, the inclusion of RNP in primary and booster immunizations was responsible for an ≈100-fold difference in the mean serum anti-KLH titers. Moreover, variation in the titers seen in different mice was minimized in the RNP plus KLH group. As shown in Fig. 7*B*, analysis of the KLH-specific proliferative response of T cells from the spleens of these twice-immunized mice revealed that RNP also enhanced this parameter of immunity.

DISCUSSION

Clearly, oral administration of rabies RNP stimulates systemic T cells and can prime animals for a rabies virus-specific neutralizing antibody response upon booster immunization with inactivated rabies virus. It is anticipated that primed RNP-specific T cells are central to the booster immunization effect described here as such T cells have a significant response advantage over their nonimmune counterparts, although it is conceivable that glycoprotein-specific T cells may become involved during the response to vaccine. The

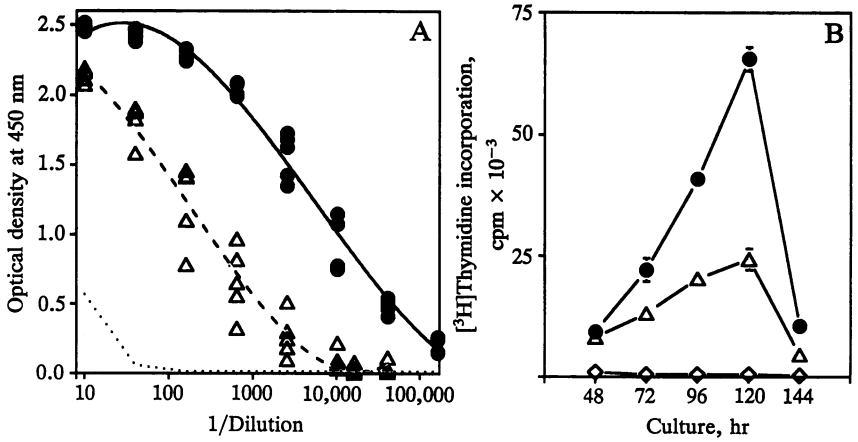

FIG. 7. Effect of RNP on the humoral (*A*) and T-cell proliferative (*B*) response to KLH. Groups of five mice were immunized i.m. with KLH (△) or a mixture of KLH plus RNP (●). (*A*) KLH-specific ELISA of serum obtained after two immunizations, where the values obtained with nonimmune serum are shown by the dotted line and third-order linear regression analysis of the results of the immunized groups is represented by the solid and dashed lines. T cells isolated from the pooled spleens of mice immunized twice i.m. with KLH (△) or a mix of RNP plus KLH (●) or left nonimmune (◇) were cultured with irradiated autologous spleen cells and 10 μg of KLH per ml (see text). (*B*) Proliferation measured by [3H]thymidine incorporation (mean cpm ± SD) at the indicated time points. In each case, the [3H]thymidine incorporation of cultures without added antigen was <1000 cpm and is not shown.

most likely mechanism is that the N-protein-specific T cells primed by the administration of RNP are furnishing help to glycoprotein-specific B cells when intact virus and RNP determinants are presented by the B cells. Alternatively, it is conceivable that the production of factors by primed RNP-specific T cells may enhance the priming and function of local glycoprotein-specific T cells early in the response to intact virus.

Though the relative contributions of RNP- and glycoprotein-specific T-helper cells toward immunity to rabies virus are open to question, it is evident that RNP is a potent oral immunogen for T cells. Primed rabies virus-specific T cells could be detected from spleen after a single p.o. dose of RNP. A second p.o. dose significantly boosted both the *in vitro* T-cell response and the serum anti-N IgA antibody levels. Two doses also greatly enhanced specific IgG antibody elicited by i.m. immunization.

The presence of N-specific IgA antibodies in the serum after p.o. administration of RNP suggests that a mucosal immune response may have been elicited. If this is the case, the elevated anti-viral antibody response to i.p. challenge with inactivated rabies virus seen after p.o. immunization with RNP perhaps demonstrates the capacity of RNP-specific T cells primed in a mucosal response to subsequently help systemic immunity.

When delivered p.o., most noninvasive and nonreplicating antigens not only are poorly immunogenic but also are likely to induce an antigen-specific systemic hyporesponsiveness termed oral tolerance (4–6). The mechanism of oral tolerance is not fully understood but appears to be due to a deficit in systemic T-cell help (22, 23). Thus, despite the fact that oral vaccines are desirable for their ease of administration, few exist. Whatever property confers upon RNP the ability to mediate a systemic response after ingestion is evidently somewhat unusual. While we have observed that rabies RNP as well as rabies virus and N protein have the capacity to stimulate nonimmune mouse T cells *in vitro* (data not shown), these responses are relatively slow to develop and weak when compared with conventional superantigens (Fig. 5). These findings argue that rabies RNP may have an intrinsic heightened ability to stimulate the immune response irrespective of any capacity to act as a superantigen.

Our experiments indicate that the structure of RNP may be an important aspect of its ability to stimulate by the oral route. N protein, which evidently contains the T-cell determinants of the RNP complex (10, 14), is not as effective as RNP when both are administered p.o. (data not shown). It is noteworthy that cholera toxin B subunit, a known enhancer of the mucosal immune response (19, 20), had little effect on p.o. immunization with RNP. We conclude that rabies RNP is sufficiently stimulatory to promote strong immunity whether given by oral or parenteral routes.

The fact that the simultaneous i.m. administration of RNP with different peptide and protein antigens augments humoral responses to the antigens, as well as *in vitro* antigen-specific T-cell proliferative responses, indicates that RNP may inherently have the ability to function as an adjuvant, possibly through an effect on the APC–T-cell interaction. We believe that this capacity to enhance responses to associated antigens is relevant to the mode of action of RNP in promoting oral immunity.

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1. Baer, G. M. (1988) *Rev. Infect. Dis.* **10**, 5644–5646.
2. Rupprecht, C. E., Wiktor, T. J., Johnston, D. H., Hamir, A. N., Dietzschold, B., Wunner, W. H., Glickman, L. T. & Koprowski, H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7947–7950.
3. Brochier, B., Kieny, M. P., Costy, F., Coppens, P., Bauduin, B., Lecocq, J. P., Lariquet, B., Chappuis, G., Desmettre, P., Afiademanyo, K., Libois, R. & Pastoret, P.-P. (1991) *Nature (London)* **354**, 520–522.
4. Chase, M. W. (1946) *Proc. Soc. Exp. Biol.* **61**, 257–259.
5. Thomas, H. C. & Parrott, D. V. M. (1974) *Immunology* **27**, 631–639.
6. Tomasi, T. B. (1980) *Transplantation* **29**, 353–356.
7. Rupprecht, C. E., Dietzschold, B., Campbell, J. B., Charlton, K. M. & Koprowski, H. (1992) *J. Wildl. Dis.* **28**, 629–635.
8. Fu, Z. F., Rupprecht, C. E., Dietzschold, B., Saikumar, P., Niu, H. S., Babka, I., Wunner, W. H. & Koprowski, H. (1993) *Vaccine* **11**, 925–928.
9. Dietzschold, B., Wang, H., Rupprecht, C. E., Celis, E., Tollis, M., Ertl, H., Heber-Katz, E. & Koprowski, H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 9165–9169.
10. Ertl, H. C. J., Dietzschold, B., Gore, M., Celis, E., Otvos, L. & Koprowski, H. (1989) *J. Virol.* **63**, 2885–2892.
11. Dietzschold, B., Rupprecht, C. E., Tollis, M., Lafon, M., Mattei, J., Wiktor, T. J. & Koprowski, H. (1988) *Rev. Infect. Dis.* **10**, 5785–5797.
12. Wiktor, T. J., Dietzschold, B., Leamnson, R. N. & Koprowski, H. (1977) *J. Virol.* **21**, 626–635.
13. Schneider, L. G., Dietzschold, B., Dierks, R. E., Mathaeus, W., Enzman, P. J. & Strohmaier, K. (1973) *J. Virol.* **11**, 748–755.
14. Fu, Z. F., Dietzschold, B., Schumacher, C., Wunner, W. H., Ertl, H. C. J. & Koprowski, H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2001–2005.
15. Liu, F. T., Zinnecker, M., Hamaoka, T. & Katz, D. H. (1979) *Biochemistry* **18**, 690–697.
16. Wiktor, T. J., MacFarlan, R. I., Foggin, C. M. & Koprowski, H. (1984) *Dev. Biol. Stand.* **57**, 199–211.
17. Burtles, S. S., Taylor, R. B. & Hooper, D. C. (1990) *Eur. J. Immunol.* **20**, 1273–1279.
18. Liang, X., Lamm, M. E. & Nedrud, J. G. (1988) *J. Immunol.* **141**, 1495–1501.
19. Vajdy, M. & Lycke, N. Y. (1992) *Immunology* **75**, 488–492.
20. Lafon, M., Lafage, M., Martinez-Arends, A., Ramirez, R., Vuillier, F., Charron, D., Lotteau, V. & Scott-Algara, D. (1992) *Nature (London)* **358**, 507–510.
21. Labrecque, N., Thibodeau, J. & Sékaly, R.-P. (1993) *Res. Immunol.* **144**, 175–180.
22. Hoyne, G. F., Callow, M. G., Kuhlman, J. & Thomas, W. R. (1993) *Immunology* **78**, 534–540.
23. Melamed, D. & Friedman, A. (1993) *Eur. J. Immunol.* **23**, 935–942.