Mucosal and systemic adjuvant activity of alphavirus replicon particles

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Communicated by Charles M. Rice, The Rockefeller University, New York, NY, January 11, 2006 (received for review October 11, 2005)

Vaccination represents the most effective control measure in the fight against infectious diseases. Local mucosal immune responses are critical for protection from, and resolution of, infection by numerous mucosal pathogens. Antigen processing across mucosal surfaces is the natural route by which mucosal immunity is generated, as peripheral antigen delivery typically fails to induce mucosal immune responses. However, we demonstrate in this article that mucosal immune responses are evident at multiple mucosal surfaces after parenteral delivery of Venezuelan equine encephalitis virus replicon particles (VRP). Moreover, coinoculation of null VRP (not expressing any transgene) with inactivated influenza virions, or ovalbumin, resulted in a significant increase in antigen-specific systemic IgG and fecal IgA antibodies, compared with antigen alone. Pretreatment of VRP with UV light largely abrogated this adjuvant effect. These results demonstrate that alphavirus replicon particles possess intrinsic systemic and mucosal adjuvant activity and suggest that VRP RNA replication is the trigger for this activity. We feel that these observations and the continued experimentation they stimulate will ultimately define the specific components of an alternative pathway for the induction of mucosal immunity, and if the activity is evident in humans, will enable new possibilities for safe and inexpensive subunit and inactivated vaccines.

vaccine vector | Venezuelan equine encephalitis virus | viral immunology | RNA virus

The control of a number of important infectious diseases by immunization is arguably one of the most significant accomplishments of the 20th century (1). However, other infectious diseases remain intractable, causing devastating morbidity and mortality in human populations, especially in resource-poor countries. Control of these diseases will depend on an expanded array of affordable and effective vaccine technologies, such as propagative and nonpropagative expression vectors based on viral and bacterial genomes. One such technology uses replicon particles based on the alphavirus Venezuelan equine encephalitis virus (VEE). VEE replicon particles (VRP) are potent inducers of antigen-specific immune responses and/or protection after pathogen or toxin challenge in various animal species including mice (2, 3), rabbits (4), cats (5), chickens (6), horses (7), guinea pigs (8), and nonhuman primates (9). Currently, VRP expressing the *gag* gene from HIV clade C are in phase-I clinical trials in the United States and Africa.

VEE virions contain a positive sense RNA genome of ≈11.5 kb. The four viral nonstructural proteins, which constitute the enzymatic activity required for RNA replication, are encoded in the 5′ two-thirds of the genome, whereas the viral structural proteins (capsid, E1, and E2) are expressed from a 26S subgenomic mRNA and encoded in the 3′ one-third of the genome (10, 11). VRP are propagation-defective viral particles carrying a modified VEE genome. The VRP system takes advantage of the high-level expression of 26S mRNA by replacing the viral structural genes with a cloned antigen gene (2). Progeny virions are not produced in VRP-infected cells, as the viral structural genes are absent from the replicon RNA; however, the replicon RNA and the mRNA encoding the antigen are expressed at high levels after infection (2, 12). To facilitate assembly of VRP, the replicon RNA is coelectroporated into permissive cells with two defective helper RNAs that lack the viral packaging signal and provide the structural genes in trans (2, 12).

VRP display a number of attractive features as vaccine delivery vehicles, including high-level antigen expression in infected cells (2), efficient *in vivo* targeting of mouse (13), and primate (A. West and R.E.J., unpublished work) dendritic cells (DCs), efficient *ex vivo* infection of human DCs (14), and safety, as the vectors are incapable of synthesizing new virion particles in infected cells (2, 12). One of the most intriguing properties of VRP is their ability to induce significant protective immunity in mucosal challenge models, even when the immunization is at a nonmucosal site (2, 6, 7, 9, 15).

The natural pathway of mucosal immune induction involves the direct delivery of immunogen to a mucosal surface and local processing of antigen in specialized aggregates of lymphoid tissue, termed mucosal inductive sites (16, 17). Stimulated lymphocytes then migrate to the corresponding mucosal surface where antigen-specific IgA and IgG are locally produced, and specific T cells reside to protect that mucosal surface from pathogen attack (18, 19). We show in this article that, unlike many vaccine vector systems that rely on mucosal delivery to access the natural inductive pathway, VRP are capable of inducing mucosal immune responses after nonmucosal delivery. Moreover, we demonstrate that this property is experimentally separable from VRP-driven immunogen production, as soluble or particulate immunogens can be simply mixed with VRP expressing an irrelevant transgene, or no transgene at all, to induce a mucosal response. Therefore, VRP exploit an alternative pathway for mucosal immune induction that is distinct from the natural pathway and suggest important applications of VRP as mucosal and systemic adjuvants in protein subunit or whole inactivated prophylactic vaccines and in immunomodulatory therapies for chronic diseases.

Results

VRP Induce Mucosal Immune Responses. Previous reports have documented the ability of peripherally inoculated VRP to induce significant protection from virulent mucosal challenge with influenza virus in mice and chickens (2, 6), simian immunodeficiency virus in macaques (9), and equine arteritis virus in horses

Conflict of interest statement: J.M.T. and R.E.J. are listed inventors on a patent application related to the subject matter of this article. R.E.J. is the unpaid Executive Director of Global Vaccines, Inc., a not-for-profit company that holds a license to the technology described in the article.

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Abbreviations: ASC, antibody-secreting cell; CT, cholera toxin; ELISPOT, enzyme-linked immunospot assay; HA, hemagglutinin; I-Flu, inactivated influenza virus; OVA, ovalbumin; VEE, Venezuelan equine encephalitis virus; VRP, VEE replicon particles; IU, infectious units.

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www.pnas.org/cgi/doi/10.1073/pnas.0600287103

(7). Also, results obtained with intranasal influenza virus challenge of hemagglutinin (HA)-VRP-immunized mice showed significantly decreased influenza virus replication in the nasal epithelium, as determined by influenza-specific plaque assay and *in situ* hybridization. (N.L.D., K. Brown, E.M.B.R., A. West, and R.E.J., unpublished work). Although VRP induced protection of the mucosal tissue, it was not directly determined whether local mucosal immune responses contributed to the observed protection. Typically, mucosal immunity is induced only when antigens are processed and presented across mucosal surfaces (20); however, VRP induced protection in these mucosal challenge models after immunization by a nonmucosal route.

We wanted to determine whether nonmucosal VRP delivery resulted in the induction of locally produced, mucosal immunity. Groups of female BALB/c mice were immunized in the rear footpad at weeks 0 and 4 with diluent, 10^5 infectious units (IU) of HA-VRP or 10 μg of formalin-inactivated influenza virus (I-Flu), as a non-VRP-vectored influenza antigen. Another group of animals was immunized in the rear footpad with 10 μg of I-Flu mixed with 10^5 IU of GFP-VRP, as an irrelevant VRP control. At various times after the second inoculation (days 3, 7, 10, 14, 18, 21, and 28), groups of three animals were killed, and the nasal mucosa were harvested for analysis in a lymphoid culture assay originally developed by Cebra and colleagues (21). Detection of flu-specific antibody in supernatant fluids from *ex vivo* nasal epithelium organ cultures was used as a measure of mucosal immune induction. Significant antibody production was not observed in supernatants from nasal epithelium until day 7 postboost and was detectable from day 7 to day 28 postboost. In comparing nasal antibody production across the range of time points, we found that VRP-containing inocula induced a statistically significant increase in flu-specific IgA antibodies in organ cultures from the nasal epithelium, compared with cultures from animals inoculated with I-Flu alone (HA-VRP compared with I-Flu, $P < 0.001$; GFP-VRP + I-Flu compared with I-Flu, $P < 0.001$, data not shown). Shown in Fig. 1 is the day-21 time point. All three antigen delivery methods were capable of stimulating local flu-specific IgG antibody production in nasal mucosa as detected in the *ex vivo* supernatants, although VRP-induced responses were significantly increased compared with responses induced by delivery of I-Flu alone (Fig. 1*A*). HA-VRP and the delivery of I-Flu mixed with GFP-VRP, but not delivery of I-Flu alone, induced flu-specific, mucosal IgA antibodies (Fig. 1*B*). Also, VRP induced a statistically significant increase in flu-specific IgG and IgA antibodies present in nasal washes of immunized animals compared with inoculation of I-Flu alone (data not shown). These results indicate that (*i*) VRP are capable of inducing local, antigen-specific antibody production in mucosal tissues after nonmucosal delivery, (*ii*) mucosal immune induction is a property of VRP, as antigen alone fails to induce significant mucosal IgA responses, and (*iii*) VRP are capable of inducing mucosal immunity either when the immunogen is expressed by the VRP, or when the immunogen is simply mixed with an irrelevant VRP that appears to serve as an adjuvant.

The mucosal response observed in the nasal epithelium did not result from an inordinately high systemic response in the VRP-containing groups. The experimental system was designed such that the systemic IgG response induced in I-Flu-immunized animals, as measured by flu-specific IgG antibodies in *ex vivo* spleen cultures, was statistically equivalent to the systemic responses induced by VRP-containing inocula (Fig. 1*C*). Therefore, any differences in the mucosal responses could not simply be attributed to higher immune responses in general. However, HA-VRP and I-Flu mixed with GFP-VRP induced significantly greater levels of flu-specific, systemic IgA antibodies than I-Flu alone, as measured in spleen culture supernatant fluids (Fig. 1*D*). Preliminary results with analogous vectors based on Girdwood virus and A.R.86 virus, alphaviruses in the Sindbis group, also suggest induction of mucosal immune responses (J.M.T., A.C.W., and M. Heise, unpublished work).

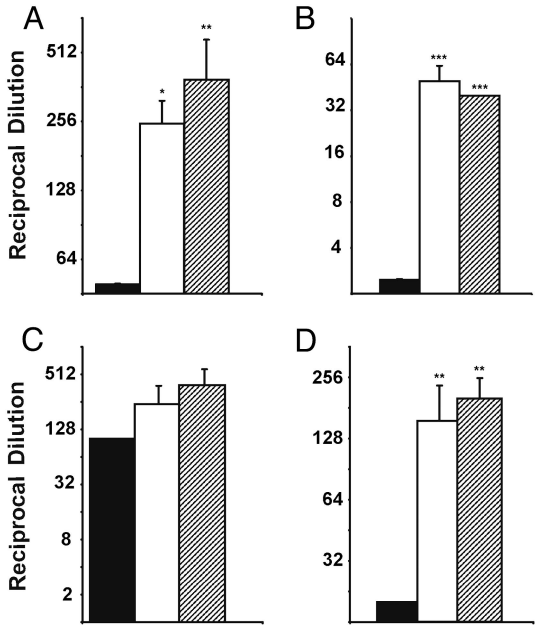

Fig. 1. VRP induce mucosal immune responses. Groups of animals were immunized in the rear footpad with diluent, 10 μg of I-Flu (solid bars), 10^5 IU of HA-VRP (open bars), or 10 μg of I-Flu plus 10^5 IU of GFP-VRP (hatched bars) at weeks 0 and 4. Three weeks after the second inoculation, lymphoid organ cultures were established from the nasal epithelium (*A* and *B*) and spleen (*C* and *D*). Culture supernatants were evaluated for flu-specific IgG (*A* and *C*) and IgA antibodies (*B* and *D*) by ELISA. Data are presented as the geometric mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with I-Flu alone, as determined by ANOVA.

VRP Possess Systemic and Mucosal Adjuvant Activity. The results reported in Fig. 1 strongly suggest that VRP themselves, independent of the expressed gene, are capable of serving as both a systemic and mucosal adjuvant after nonmucosal delivery. To confirm this hypothesis, groups of eight animals were immunized in the rear footpad with 10^6 IU of VRP not expressing any transgene (null VRP) mixed with either 0.1 or 1.0 μg of I-Flu at weeks 0 and 4. Although null VRP do not express an inserted gene behind the 26S promoter, a short 175-nt noncoding mRNA is predicted from the sequence. Animals were bled 2 weeks postboost, and flu-specific serum IgG antibodies were analyzed by ELISA. As shown in Fig. 2, the presence of null VRP in the inoculum increased the flu-specific systemic antibody response by up to 44-fold (1.0 μg dose of I-Flu). To assess mucosal antibody responses, fecal extracts were prepared and analyzed for the presence of flu-specific mucosal antibodies by ELISA (Fig. 2 *B* and *C*). Antibodies present in fecal extracts are almost exclusively locally produced, with minimal contribution from serum-derived antibodies (22). Flu-specific fecal IgA antibodies were barely detectable after immunization with I-Flu alone; however, the inclusion of null VRP as an adjuvant augmented those responses by ≈60 fold (1.0-μg dose of I-Flu, IgA). These data confirm that VRP possess systemic and mucosal adjuvant activity for a particulate antigen.

To further characterize the adjuvant properties of VRP, the following experiments used null VRP and a soluble test antigen, ovalbumin (OVA), rather than a particulate antigen (I-Flu). Groups of six female BALB/c mice were immunized at weeks 0 and 4 with 10 μg of OVA, either alone or coinoculated with 10^6 IU of null VRP, by both parenteral (footpad) and mucosal

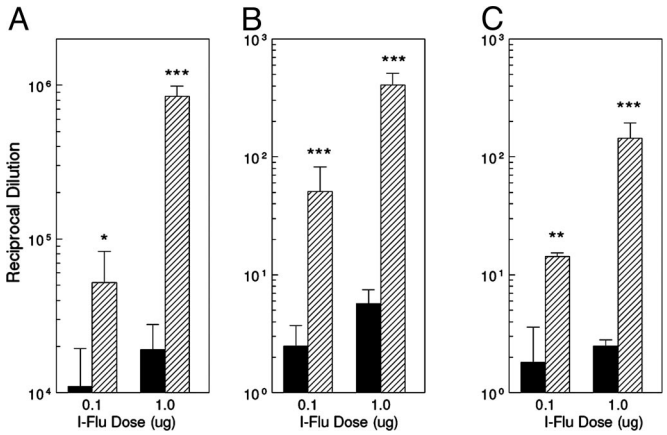

Fig. 2. VRP adjuvant activity for particulate antigens. Groups of eight animals were immunized in the rear footpad with 0.1 or 1.0 μg of I-Flu in the presence (hatched bars) or absence (solid bars) of 10^6 IU of null VRP at weeks 0 and 4. Two weeks after the second inoculation, flu-specific IgG antibodies were measured in sera (*A*) and fecal extracts (*B*), and flu-specific IgA antibodies were measured in fecal extracts (*C*) by ELISA. Data are presented as the geometric mean ± SEM. *, $P < 0.02$; **, $P < 0.005$; ***, $P < 0.0003$ compared with I-Flu alone, as determined by Mann–Whitney.

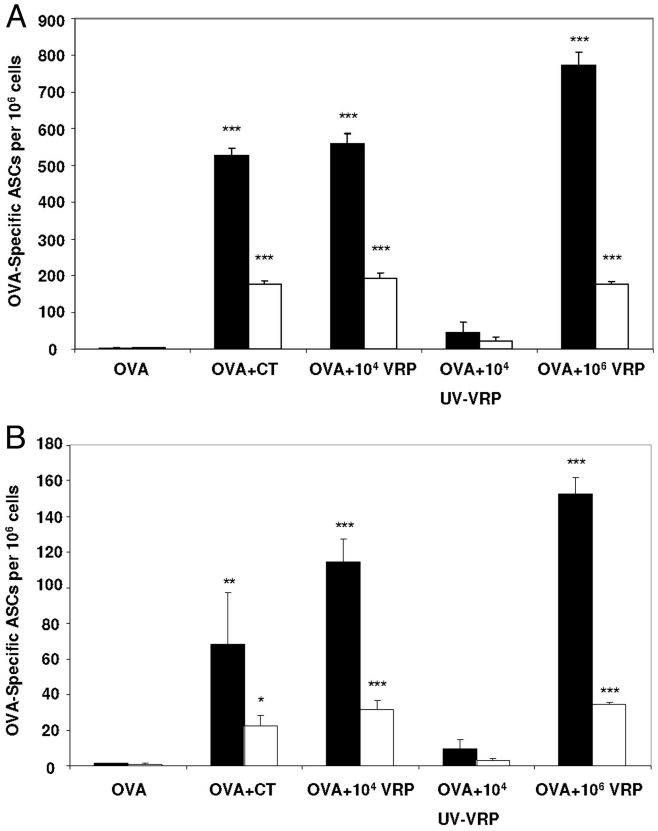

Fig. 3. Systemic and mucosal adjuvant activity of UV-treated VRP. Groups of six animals were immunized in the rear footpad with 10 μg of OVA alone or coinoculated with 1.0 μg of CT, 10^4 IU of null VRP, 10^4 IU of UV-VRP, or 10^6 IU of null VRP at weeks 0 and 4. One week after the second inoculation, splenocytes (open bars) and nasal lymphocytes (solid bars) were isolated from immunized animals and analyzed for the presence of OVA-specific IgG-secreting cells (*A*) and IgA-secreting cells (*B*) by ELISPOT. Data are presented as the geometric mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with OVA alone, as determined by ANOVA.

(intranasal) delivery. As shown in Fig. 5, which is published as supporting information on the PNAS web site, both footpad and nasal delivery of OVA alone resulted in detectable OVA-specific serum IgG titers 3 weeks postboost. The coinoculation of null VRP with OVA increased OVA-specific serum IgG responses by ≈60- and 1,400-fold after footpad and nasal delivery, respectively. To assess mucosal antibody responses, fecal extracts were prepared from vaccinated animals before the booster inoculation and at weeks 1, 2, and 3 postboost, and analyzed for the presence of OVA-specific mucosal IgG and IgA antibodies by ELISA. Delivery of OVA alone failed to consistently induce detectable levels of OVA-specific fecal antibodies over background after either footpad or nasal immunization 3 weeks postboost. However, the inclusion of null VRP in the inoculum resulted in an ≈20- to 60-fold increase in OVA-specific fecal IgG and IgA antibody titers (Fig. 5 *B* and *C*), regardless of the route of immunization. Taken together, the observations using I-Flu and OVA confirm the systemic and mucosal adjuvant activity of VRP after either mucosal or nonmucosal delivery of soluble or particulate immunogens.

VRP RNA Replication Is a Trigger for Adjuvant Activity/Immune Induction. The critical VRP-specific parameters that mediate adjuvant activity are currently undefined. Numerous molecular sensors are capable of recognizing viral products in virus-infected cells (23), including members of the toll-like receptor family (24, 25), and a number of IFN-inducible proteins (26, 27). We hypothesize that one or more of these pathways might be involved in recognizing RNA products produced after VRP infection and might play a critical role in VRP adjuvant activity. To test the hypothesis that VRP RNA replication is necessary for adjuvant activity, we treated null VRP with UV light before inoculation. UV treatment causes the formation of uridine dimers in the replicon RNA, which blocks both RNA replication and translation of the input RNA, and allows evaluation of replication-defective VRP as molecular adjuvants.

Groups of six BALB/c mice were inoculated in the rear footpad at weeks 0 and 4 with 10 μg of OVA alone or 10 μg of OVA mixed with (*i*) 1.0 μg of cholera toxin (CT), a known systemic and mucosal adjuvant used here as a positive control (28), (*ii*) 10^4 IU of null VRP, (*iii*) 10^4 IU of null VRP treated with UV light (UV-VRP), or (*iv*) 10^6 IU of null VRP. At 1 week postboost, serum was harvested from immunized animals and analyzed for the presence of OVA-specific IgG antibodies by ELISA. OVA-specific serum IgG titers were increased by ≈64- and 114-fold after the codelivery of OVA plus 10^4 or 10^6 IU of VRP, respectively (Table 1, which is published as supporting information on the PNAS web site). In contrast, codelivery of OVA and 10^4 IU of UV-VRP failed to induce a statistically significant increase in OVA-specific serum IgG antibodies ($P > 0.05$). These results suggest that viral RNA replication was required for the immune stimulation observed with null VRP. Importantly, the adjuvant effect of VRP was comparable to responses induced by 1.0 μg of the control adjuvant, CT, under these conditions.

To quantitate the number of OVA-specific IgG- and IgA-secreting cells in spleen and nasal epithelium of the same animals, single-cell suspensions were prepared and analyzed in an antibody-secreting cell (ASC) enzyme-linked immunospot assay (ELISPOT). Increased levels of IgG (Fig. 3*A*) and IgA (Fig. 3*B*) ASCs were present in spleen and nasal epithelium in the OVA-plus-VRP inoculated animals, compared with the OVA-alone group, again demonstrating a clear systemic and mucosal VRP adjuvant activity leading to the local production of antigen-specific antibodies in both systemic and mucosal tissues. UV treatment of VRP before inoculation largely abrogated this effect, indicating the importance of VRP RNA function and also suggesting that contaminants potentially present in the VRP

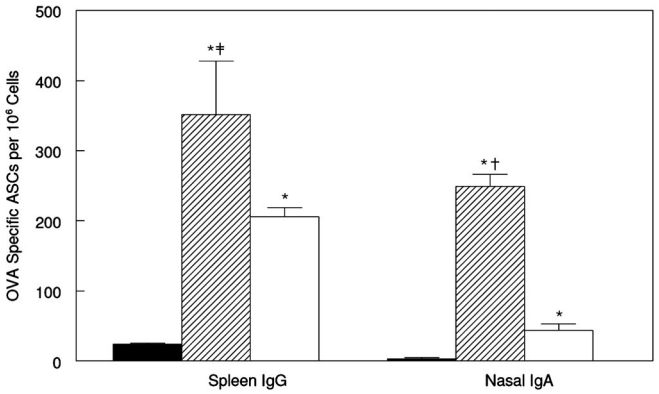

Fig. 4. Systemic and mucosal adjuvant activity of VRP compared with CpG DNA. Groups of eight animals were immunized in the rear footpad with 10 μg of OVA alone (solid bars) or coinoculated with 10^5 IU of null VRP (hatched bars) or 1.0 μg of CpG DNA (open bars) at weeks 0 and 4. Two weeks after the second inoculation, splenocytes were isolated and analyzed for the presence of OVA-specific IgG ASCs, and nasal lymphocytes were isolated and analyzed for the presence of OVA-specific IgA ASCs by ELISPOT. Data are presented as the geometric mean ± SEM. *, $P < 0.001$ compared with OVA alone; †, $P < 0.01$ compared with CpG; ‡, $P < 0.05$ compared with CpG.

preparations (such as LPS) were not responsible for the observed adjuvant activity. Again, VRP adjuvant activity as measured by ASC ELISPOT was comparable with that of CT. These results demonstrate that null VRP can act as a true mucosal adjuvant, and VRP RNA replication is likely the molecular trigger for the adjuvant activity.

VRP Adjuvant Activity as Compared with Adjuvant Activity of CpG DNA. We sought to determine how the VRP adjuvant compared with another known adjuvant, CpG DNA. Unmethylated CpG motifs found in bacterial genomes are recognized by the innate immune system through interactions with TLR9 and increase immunity to coimmunized antigens in numerous experimental systems (reviewed in ref. 29). To further characterize the relative strength of VRP adjuvant activity, groups of eight BALB/c mice were inoculated in the rear footpad at weeks 0 and 4 with 10 μg of OVA alone, 10 μg of OVA mixed with 10^5 IU of null VRP, or 10 μg of OVA mixed with 1.0 μg of CpG DNA. Two weeks after the second inoculation, sera, fecal extracts, and vaginal lavage samples were prepared from individual animals and analyzed for the presence of OVA-specific antibodies by ELISA. Also at 2 weeks postboost, single-cell suspensions were prepared from spleen and nasal epithelium and analyzed for OVA-specific ASCs by ASC ELISPOT. As shown in Fig. 4, both VRP and CpG augmented OVA-specific spleen IgG ASCs compared with OVA alone ($P < 0.001$ and $P < 0.05$, respectively). Although VRP adjuvanted systemic OVA responses to a greater extent than CpG, as measured by spleen ASC, measurement of OVA-specific serum IgG titers suggested that the CpG and VRP systemic adjuvant effects were comparable (Table 2, which is published as supporting information on the PNAS web site). However, VRP induced a significant adjuvant effect on mucosal IgA responses in fecal extracts and vaginal washes and in IgA ASCs in the nasal epithelium (Fig. 4 and Table 2). By each of these assays, VRP-adjuvanted OVA responses in mucosal tissues were superior to OVA plus CpG. These data suggest that the systemic adjuvant activity of VRP is at least as strong as that of CpG and that VRP possess significantly stronger mucosal adjuvant activity.

Discussion

Alphavirus replicon vectors expressing pathogen-derived immunogens have been used extensively as vaccine delivery vehicles and have proven effective at inducing significant protection from challenge with a number of important pathogens in experimental and natural hosts. However, the mechanisms that govern immune induction after vector delivery remain largely unexplored.

We demonstrate in this article that VRP possess inherent immunostimulatory properties that are independent of protein production. Either irrelevant or null VRP, simply codelivered with soluble OVA protein or inactivated influenza virions, dramatically augmented antigen-specific antibody production in both the systemic and mucosal compartments, compared with inoculation of antigen alone. In work not presented here, VRP systemic and mucosal adjuvant activity also has been demonstrated with Norwalk virus-like particles (A. LoBue, J.M.T., R. Baric, and R.E.J., unpublished work), cowpox B5R protein (N. Thornburg, J.M.T., and R.E.J., unpublished work), and simian immunodeficiency virus gp120 (A. West, J.M.T., and R.E.J., unpublished work), suggesting that the VRP adjuvant functions without respect to the antigen. In the present study we have measured only short-term immunity with VRP adjuvants. However, VRP used as expression vectors elicited responses that endured throughout the lifetime of the animal. If we assume that the immunological parameters that govern VRP as expression vectors are the same as those that govern immune induction with VRP as adjuvants, then it is likely that adjuvant-induced immunity will be equally long-lived.

We demonstrate the adjuvant property of alphavirus replicon particles for both systemic and mucosal immunity, even when administered by a nonmucosal route. A number of recent reports have identified other viral (30–33) and bacterial (34) particles that possess various types of adjuvant activity when codelivered with antigen. We speculate that such activity is also likely to play an important role in immune induction under conditions in which such particles (including VRP) are engineered as vectors to express a given immunogen. Although those other reports document the ability of microbial particles to serve as adjuvants, no other system has demonstrated mucosal immune induction after nonmucosal delivery, as is observed with VRP. It will be of interest to determine whether other viruses are capable of augmenting mucosal antibody responses after nonmucosal delivery, or if this property is unique to VEE.

The natural pathway of mucosal immune induction relies on antigen processing and presentation at mucosal surfaces and results in the local production of IgA antibodies at those surfaces (20, 35). VRP were capable of immune induction via the natural pathway, as nasal delivery resulted in the induction of mucosal immunity. However, VRP were also capable of exploiting an alternative pathway that resulted in mucosal immunity after nonmucosal inoculation. Although there have been a limited number of examples where induction of mucosal immunity occurred after inoculation at a parenteral site (reviewed in refs. 36 and 37), there is little consistency among the several examples, and none of them is analogous to the null VRP adjuvant activity described here (38–47). Likewise, induction of mucosal immunity has been demonstrated with alphavirus expression vectors, but only after immunization (48, 49) or boost (15) at a mucosal surface, and in none of these instances was the potential for mucosal adjuvant activity examined.

The mechanism by which VRP trigger mucosal immunity after nonmucosal delivery is undefined at present. One potential explanation is that either free VRP, or cells infected by VRP in the skin (13) or lymph node migrate to a traditional mucosal inductive site, such as Peyer's patches or mesenteric lymph node, and induce local antibody production (36). However, experiments using GFP-VRP have failed to consistently demonstrate VRP-infected cells in such tissues (E.M.B.R., J.M.T., and R.E.J., unpublished work). We favor the hypothesis that the lymph node draining the site of VRP inoculation develops at least some functions characteristic of a mucosal inductive site. In support of this idea, preliminary experiments demonstrate the production

of antigen-specific, multimeric IgA in the draining lymph node (DLN) in response to inoculation of VRP (J.M.T. and R.E.J., unpublished work). It needs to be determined whether additional characteristics of a true mucosal inductive site are present in the DLN of VRP-inoculated mice. We feel that detailed examination of this alternative pathway for the induction of mucosal immunity in the VRP experimental system will contribute to a greater understanding of alphavirus-induced immunity, in particular, and mucosal immunity in general.

The molecular basis for the adjuvant activity likely resides in the ability of the VRP genome to replicate, given the sensitivity of adjuvant activity to UV inactivation. We suggest that an element present during virus replication is recognized in infected host cells and that this recognition initiates a cascade of events that ultimately leads to the induction of immunity to codelivered antigens. The most prominent candidates include viral RNA and/or replicative intermediates and their interactions with components of the innate immune system. A variety of cellular sentinel molecules exist, such as TLR3 (24), TLR7 (25), Rig-I, MDA-5 (27), protein kinase R, and RNaseL (26), which are capable of recognizing viral replicative molecules. In fact, a recent report (50) implicates RNaseL in immune induction to a tolerant melanoma antigen in an alphavirus replicon system.

Both transgene-expressing particles and particles lacking a transgene possess adjuvant activity, suggesting that adjuvant activity neither depends on, nor is inhibited by, the presence of a particular transgene protein. The VRP constructs lacking a transgene are predicted to express a short, noncoding RNA. It is unlikely that this truncated subgenomic RNA, or the presence or activity of the 26S promoter itself, is responsible for the observed adjuvant activity. Another formal possibility is that translation of the replicase proteins is responsible for the activity.

One potential trivial explanation for the adjuvant effect is that it is mediated by a contaminant present in VRP preparations (such as LPS). However, two observations strongly suggest that a contaminant is not the predominant mechanism of immune activation: (*i*) no adjuvant activity was observed after codelivery of identically treated media from a mock VRP preparation (data not shown), and (*ii*) UV treatment of VRP ablated adjuvant activity.

We have compared VRP adjuvant activity to that of CT and CpG DNA. Results from such comparisons suggest that systemic responses induced by VRP are at least equivalent to that of both CT and CpG DNA. Moreover, after nonmucosal delivery VRP mucosal adjuvant activity appears to be comparable to that of CT and superior to CpG DNA. A number of important questions regarding VRP adjuvant activity remain to be answered, such as how VRP-induced systemic and mucosal immune responses compare with those of other peripherally delivered adjuvants, such as alum, and mucosally delivered CT and whether VRP act as a systemic and mucosal T cell adjuvant. These additional comparisons will allow more accurate evaluations of the relative efficiency of VRP-induced immune stimulation.

In summary, we have demonstrated two activities of alphavirus-derived viral vectors: (*i*) induction of local mucosal immune responses after inoculation at a remote, nonmucosal site and (*ii*) systemic and mucosal adjuvant activity with codelivered soluble and particulate immunogens. We feel that these observations and the continued experimentation they stimulate will advance a search for adjuvant activity among other viruses and viral vectors, will ultimately define the specific components of an alternative pathway for the induction of mucosal immunity, and if the activity is evident in humans, will enable new possibilities for safe and inexpensive subunit and inactivated vaccines.

Materials and Methods

VEE Replicon Constructs. The construction and packaging of VRP have been described (2, 51). The replicon constructs used in this study were (*i*) replicons expressing GFP (GFP-VRP), (*ii*) replicons expressing the HA gene from influenza virus (HA-VRP), and (*iii*) replicons that lack a functional transgene downstream of the 26S promoter (null VRP). Null VRP contain the viral nonstructural genes, 14 nt of VEE sequence downstream of the 26 mRNA transcription start site, an inserted 43-nt-long multiple cloning site, and the 118-nt 3′ UTR. All replicon particles used in this study were packaged in the wild-type (V3000) envelope (2).

Animals and Immunizations. Seven- to 8-week-old female BALB/c mice were immunized either in the rear footpad or intranasally at weeks 0 and 4. Grade V chicken egg albumin (OVA) was purchased from Sigma, CT was purchased from List Biological Laboratories (Campbell, CA), and CpG DNA (ODN 1826) was purchased from Invivogen (Montreal). Formalin-I-Flu (Charles River Laboratories) was dialyzed against PBS in a Slidalyzer cassette (Pierce) according to the manufacturer's guidelines before immunization.

Inactivation of VRP by UV Treatment. Null VRP preparations were diluted to a concentration of 10^6 units/ml, and 0.2-ml aliquots were placed in individual wells in a 48-well tissue culture plate. The plates were exposed to a UV lamp (Sun-Kraft, Chicago) at a distance of 5 cm for 20 min. No VRP-infected cells were detectable *in vitro* after infection of baby hamster kidney cells with undiluted UV-VRP (data not shown).

Sample Collection. Animals were bled either from the tail vein or after cardiac puncture, and sera were analyzed by ELISA (see below). Preparation of fecal extracts was modified from Bradney *et al.* (52). Vaginal lavage was performed by washing the exterior vaginal opening with 0.07 ml of PBS 8–10 times.

Lymphoid Organ Cultures. Lymphoid cultures, originally developed by Cebra and colleagues (21), were modified from Coffin *et al.* (53). Briefly, spleen and nasal tissue were dissected from immunized animals and washed three times by aspiration and resuspension. Nasal tissue from individual animals was placed in a well of a 48-well tissue culture plate containing 0.3 ml of media and incubated at 37°C for 7 days at which time supernatants were harvested.

ELISA. ELISAs for influenza- and OVA-specific antibodies were performed according to standard ELISA methods (2). Antibody endpoint titers are reported as the reciprocal of the highest dilution that resulted in an $OD_{450} \geq 0.2$. In lymphoid culture supernatants, endpoint titers for flu-specific IgA are reported as the reciprocal of the highest dilution that results in an OD_{450} reading at least 2 SDs greater than values obtained from mock-vaccinated animals.

ASC ELISPOT. Single-cell suspensions were prepared from both spleen and nasal epithelium. Whole spleens were disrupted between frosted glass slides, and red blood cells were lysed after addition of ammonium chloride buffer. Cells were washed and placed on a Lympholyte-M density gradient. Banded cells were harvested, washed, and counted. For preparation of nasal lymphocytes, nasal tissue from the tip of the nose to just anterior of the eye sockets was harvested from immunized animals, and the upper palate, including the nasal-associated lymphoid tissue, was carefully removed. Nasal tissue was physically disrupted and incubated at 37°C for 2 h in complete media containing Collagenase A, DNase I, and glass beads. After digestion, cells were filtered, washed,

resuspended in 44% Percoll, and layered on Lympholyte-M as described for spleen cells above. Banded cells were harvested, washed, and counted. Cells were pooled from two animals. ASC ELISPOT analysis was modified from previous reports (54, 55).

Statistical Analysis. Antibody titers and ASC numbers were evaluated for statistically significant differences by either the ANOVA or Mann–Whitney tests (INSTAT; GraphPad, San Diego). $P \leq 0.05$ was considered significant.

Additional Methods. See *Supporting Text*, which is published as supporting information on the PNAS web site, for more detailed methods.

We thank Mark Heise and Clayton Beard for critical review of the manuscript, members of the Carolina Vaccine Institute for helpful discussions, and Dwayne Muhammad for excellent technical assistance. This work was supported by National Institutes of Health Grants P01-AI46023 and R01-AI51990 (to R.E.J.).

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