Immunopotentiating Reconstituted Influenza Virus Virosome Vaccine Delivery System for Immunization against Hepatitis A

R. Glück, R. Mischler, S. Brantschen, M. Just,* B. Althaus, and S. J. Cryz, Jr.
*Swiss Serum and Vaccine Institute, CH-3001 Berne, Switzerland; and *Department of Pediatrics, University Children's Hospital, CH-4000 Basel, Switzerland*

Abstract

Hepatitis A virus (HAV) was purified from MRC-5 human diploid cell cultures, inactivated with formalin, and evaluated for safety and immunogenicity in humans. Three vaccine formulations were produced: (*a*) a fluid preparation containing inactivated HAV, (*b*) inactivated HAV adsorbed to $Al(OH)_3$, and (*c*) inactivated HAV coupled to novel immunopotentiating reconstituted influenza virosomes (IRIV). IRIV were prepared by combining phosphatidylcholine, phosphatidylethanolamine, phospholipids originating from the influenza virus envelope, influenza virus hemagglutinin, and neuraminidase. The HAV–IRIV appeared as unilamellar vesicles with a diameter of ~ 150 nm when viewed by transmission electron microscopy. Upon intramuscular injection, the alum-adsorbed vaccine was associated with significantly ($P < 0.01$) more local adverse reactions than either the fluid or IRIV formulations. 14 d after a single dose of vaccine, all the recipients of the IRIV formulation seroconverted (≥ 20 mIU/ml) versus 30 and 44% for those who received the fluid and alum-adsorbed vaccines, respectively ($P < 0.001$). The geometric mean anti-HAV antibody titer achieved after immunization with the IRIV-HAV vaccine was also significantly higher ($P < 0.005$) compared with the other two vaccines. (*J. Clin. Invest.* 1992. 90:2491–2495.) Key words: adjuvant • immunogenicity • antibody • hepatitis A • vaccine

Introduction

Numerous vaccines composed of synthetic peptides, purified subunit antigens, or small inactivated virus are currently undergoing evaluation (1–3). Although such vaccines offer the benefit of being antigenically defined and safe due to the absence of toxic contaminating substances, many are poorly immunogenic due to such innate attributes as molecular size and restricted epitope recognition (4, 5). New adjuvants or delivery systems are needed that will render such antigens highly immunogenic if they are to become effective vaccines (6, 7).

A number of approaches have recently been undertaken to address this issue including the synthesis of immunostimulating complexes (8), proteosomes (9), liposomes (8, 10, 11), conjugates (12), surface-active agents (8), monophosphoryl lipid A (5, 13), muramyl dipeptide derivatives (13), and cytokines (14). The incorporation of viral membrane proteins into liposomes has also been shown to potentiate the immune response to several viral glycoproteins. Such virosome vaccines have been constructed using influenza (15), rabies (16), and herpes simplex viruses (17) with promising results.

In an effort to develop an antigen delivery system with adjuvanting activity, we have synthesized novel unilamellar structures composed of phosphatidylethanolamine (PE),[1] phosphatidylcholine (PC), and the hemagglutinin (HA), neuraminidase, and phospholipids from influenza virus termed immunopotentiating reconstituted influenza virosomes (IRIV). All constituents were selected on the basis of potential adjuvant effects or prior documented safety when parenterally administered to humans. Therefore, the HA functions to (*a*) bind sialic acid–containing receptors on the surface of macrophages and other immunocompetent cells (18); (*b*) mediate the membrane fusion of the IRIV to cells facilitating antigen delivery (19); and (*c*) serve as a "recognition antigen" since most humans can be considered "primed" to HA due to prior exposure through disease or vaccination (20, 21). The reactive amino group of PE can serve as a site through which antigens can be covalently coupled to the IRIV, if need be.

To investigate the potential of IRIV to serve as a vaccine delivery system, inactivated hepatitis A virus (HAV) antigen was formulated into an IRIV. The safety and immunogenicity of the IRIV–HAV vaccine was compared with fluid and alum-adsorbed vaccines in seronegative adults. The IRIV vaccine was significantly more immunogenic than either of the two other vaccine formulations in addition to evoking markedly fewer and milder local reactions compared with the alum-adsorbed vaccine.

Methods

Purification and inactivation of HAV. The RG-S3 HAV strain was cultured on MRC-5 human diploid cells. The virus was purified from disrupted cells by ultrafiltration, extraction in *n*-heptane to remove lipids, 30% sucrose cushion ultracentrifugation, and $CsCl_2$ density gradient ultracentrifugation. HAV was inactivated by treatment with formalin (0.25% wt/vol) at 37°C for 10 d. Inactivation was confirmed by lack of viral replication after prolonged incubation of the formalin-treated viral concentrate on MRC-5 cells.

Formulation of vaccines. The purified HAV concentrate was filter sterilized and the HAV antigen concentration was determined by radioimmunoassay (22). HAV antigen content is expressed as radioimmunoassay units (RU). The fluid vaccine formulation was prepared by diluting the sterile concentrate to 2,000 RU/ml in sterile physiological

Address correspondence and reprint requests to Dr. R. Glück, Department of Virology, Swiss Serum and Vaccine Institute, PO Box 2707, CH-3001 Berne, Switzerland.

Received for publication 5 March 1992 and in revised form 22 July 1992.

J. Clin. Invest.

0021-9738/92/12/2491/05 $2.00
Volume 90, December 1992, 2491–2495

1. *Abbreviations used in this paper:* GMT, geometric mean titer; HA, hemagglutinin; HAV, hepatitis A virus; IRIV, immunopotentiating reconstituted influenza virosomes; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RU, radioimmunoassay units.

saline and aseptically filling into sterile vials. The alum-adsorbed vaccine was formulated by aseptically mixing an equal amount of sterile inactivated HAV (2,000 RU/ml) with 0.8% (wt/vol) $Al(OH)_3$ for 72 h at 37°C with gentle stirring. The vaccine was then aseptically aliquoted into sterile vials.

The IRIV vaccine was produced as follows. HA from the A/Singapore/6/86 strain of influenza virus was isolated as described below. Purified virus was stabilized in a buffer containing 0.1 M octaethyleneglycol mono(*n*-dodecyl)ether (Nikko Chemicals, Tokyo, Japan), 7.9 mg NaCl/ml, 4.4 mg trisodium citrate · $2H_2O$/ml, 2.1 mg 2-morpholinoethane sulfonic acid/ml, and 1.2 mg *N*-hydaxylethyl-piperazine-*N'*-2-ethane sulfonic acid/ml, pH 7.3. This mixture was centrifuged at 100,000 *g* for 30 min. The supernatant, which contained the HA, trace amounts of neuraminidase, and viral phospholipids, was saved.

PC (Sigma Chemical Co., St. Louis, MO) and PE (Sigma Chemical Co.) (75:25%; wt/wt) were suspended in 0.01 M Tris–0.1 M NaCl, pH 7.3, and homogenized. Recrystallized sodium cholate (Sigma Chemical Co.) was added to a final concentration of 0.02 M to disintegrate multilamellar structures. To this solution was added the HA-containing supernatant, and the suspension was stirred for 1 h at 4°C. The suspension was applied to a Sephadex G-50 column (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) equilibrated in 0.01 M Tris–0.1 M NaCl, pH 7.3. The sealed column was placed in a water bath. During elution ultrasonic shocks (50 kHz; 10 s/min) were passed through the water bath using an ultrasonification device (Bransonic; Branson Europe BV, The Netherlands). The void volume fractions, which contained the IRIV, were pooled and rechromatographed under identical conditions. The final IRIV suspension contained undetectable levels of cholate (< 10 cholate molecules/IRIV) and octaethyleneglycol mono(*N*-dodecyl)ether (< 150 nM). The IRIV possessed an average diameter of ~ 150 nm.

The purified, inactivated HAV suspension with a known amount of antigen was centrifuged for 4 h at 100,000 *g* to pellet the virus. An appropriate quantity of the IRIV suspension was added to the pellet and gently resuspended by shaking. The suspension was gently stirred at 20°C for 48 h to allow the HAV to adsorb onto the surface of the IRIV. This bulk suspension was diluted with sterile phosphate-buffered saline, pH 7.4, to a final concentration of 2,000 RU HAV antigen/ml and bottled.

All vaccines were tested for sterility and general safety as described in the *European Pharmacopoeia* (23).

Antigen content determination. HA was quantitated by a single radial diffusion test (24). Values were standardized using the A/Singapore/6/86 reference preparation from the National Institute of Biological Standards and Control (London, UK). HAV antigen content was determined using a solid-phase radioimmunoassay (22) and expressed as RU. A reference preparation of HAV antigen provided by the Walter Reed Army Institute of Research (Washington, D.C.) was used as a standard.

Animal immunogenicity studies. BALB/c mice (groups of 10) were immunized with one fifth of a human dose (200 RU) of the three vaccine formulations. Serum samples were obtained 4 wk later and were assayed by radioimmunoassay.

Clinical studies. The protocol was approved by the ethical committee of the Children's Hospital (Basel, Switzerland). A total of 120 HAV-seronegative (< 10 mIU/ml) healthy adults were enrolled after obtaining written informed consent. Subjects were randomized to receive either fluid, alum-adsorbed, or IRIV vaccine. The vaccine (0.5 ml) was administered intramuscularly into the deltoid region. Volunteers were observed for ~ 30 min after vaccination for immediate-type reactions. Each volunteer was asked to record all adverse reactions on a report sheet for the 4 d after immunization. Serum samples for anti-HAV antibody determinations were taken at the time of immunization and 14, 28, 180, and 352 d later.

Antibody determination. Total anti-HAV antibody content was determined using an automated microparticle enzyme immunoassay test kit (IMX HAVAB; Abbott Laboratories, North Chicago, IL) with antibody levels expressed as mIU/ml. A reference serum (World Health Organization, Geneva, Switzerland) was run in parallel with the samples.

Statistics. Statistical significance between geometric mean titers was determined using the rank-sum statistic *t* test. Difference between seroconversion and adverse reaction rates was determined by chi-square analysis.

Results

To confirm that HAV antigen was firmly associated with IRIV, samples were analyzed by sucrose density gradient ultracentrifugation (Fig. 1). Purified, inactivated HAV was pelleted when run on an isopycnic sucrose gradient. In comparison, both HAV antigen and influenza HA banded at a density of 1.158 when formulated as an IRIV. The fractions containing HAV antigen and HA were also rich in viral phospholipids. Electron photomicrographs of the IRIV–HAV vaccine showed spherical unilamellar vesicles with a mean diameter of ~ 150 nm (Fig. 2). Spike-like components (10–15 nm), which resemble those of influenza HA, could be seen protruding from the surface of the IRIV.

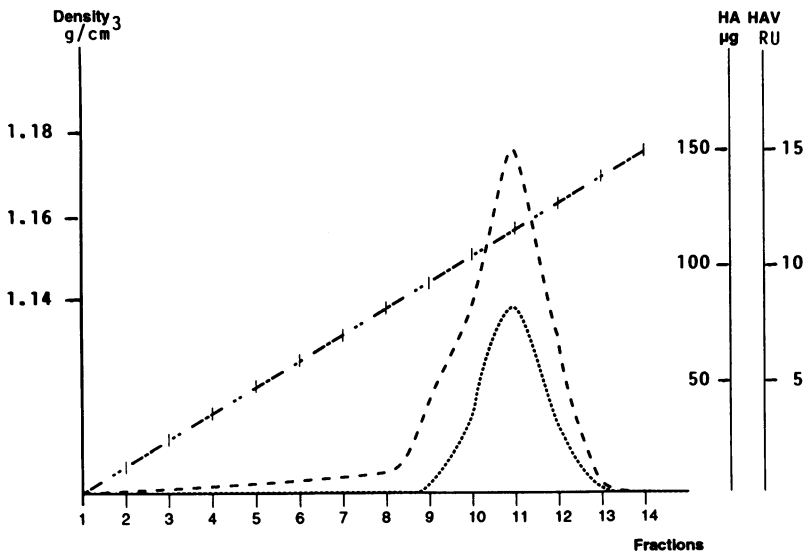

Figure 1. Isopycnic sucrose density gradient banding profile of the HAV–IRIV vaccine. Four sucrose layers in phosphate-buffered saline, pH 7.4, at concentrations of 0, 15, 25, and 45% (wt/vol) were used to prepare this gradient. (– – –) influenza HA; (· · · · ·) HAV antigen.

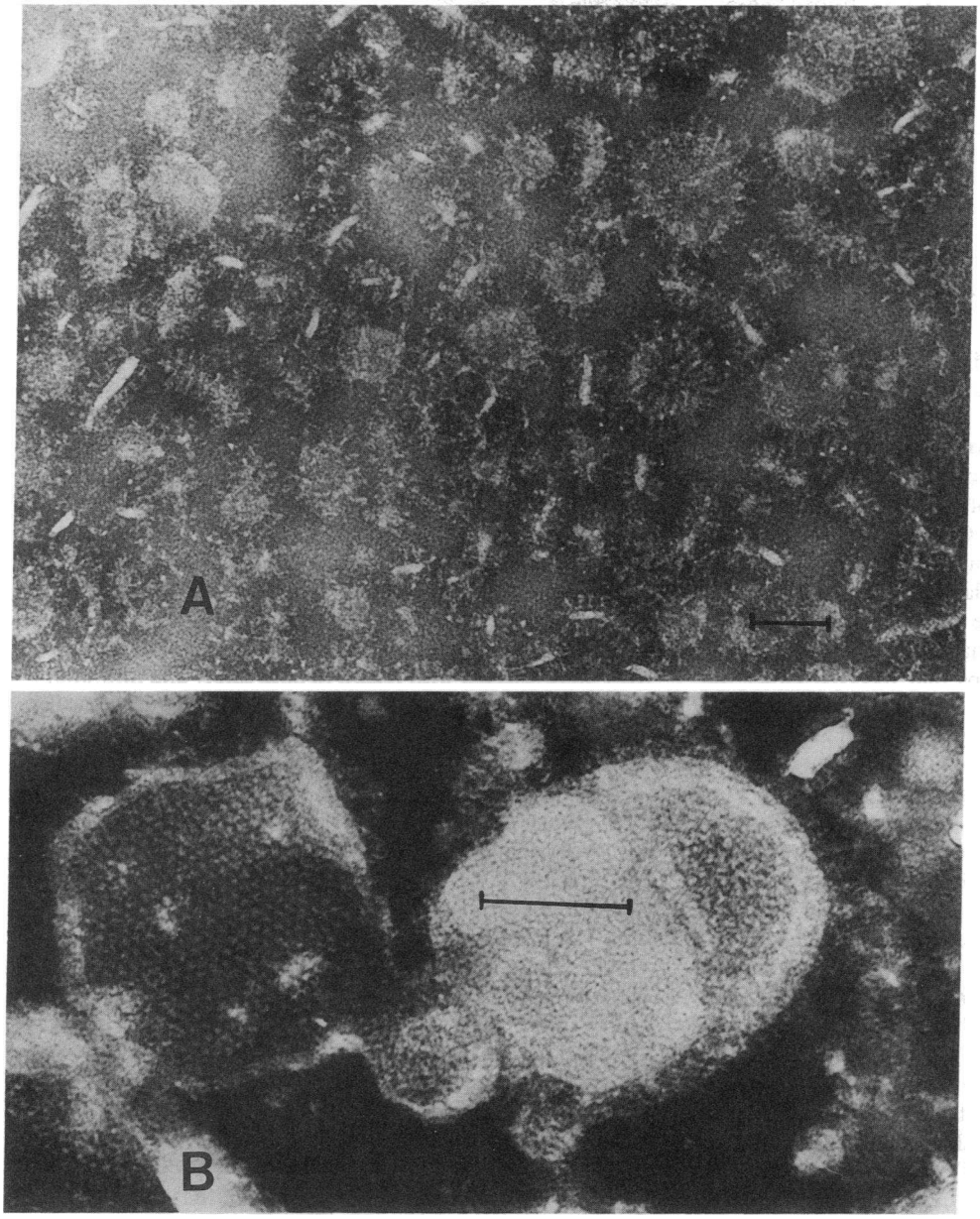

Figure 2. Transmission electron micrographs of IRIV–HAV vaccine. (*A*) ×100,000. (*B*) ×200,000. Bar, 100 nm.

Each vaccine formulation contained 1,000 RU HAV antigen/0.5-ml dose. One dose of the IRIV–HAV formulation also contained 10 μg of influenza HA and 125 μg total phospholipids. All three vaccines were found to be sterile and nontoxic for animals by standard test methods. In addition, all three formulations elicited a good anti-HAV antibody response in laboratory animals. Therefore, the geometric mean titer (range) in mIU/ml for mice immunized with fluid, alum-adsorbed, or IRIV vaccine was 278 (13–435), 521 (130–980), and 534 (121–972), respectively. There was no significant difference between these values.

Based upon the above findings, a phase I trial was initiated to evaluate the safety and immunogenicity of these vaccines in humans. Each formulation was administered intramuscularly to 40 healthy adult volunteers seronegative for HAV antibody. The groups were well matched in regard to age and sex. Adverse reactions associated with immunization are shown in Table I. Pain at the injection site was the most frequently reported complaint with all the vaccines. Such discomfort was classified as moderate by one vaccinee (2.5%) who received the fluid formulation, nine (23%) who were immunized with the alum-adsorbed vaccine, and one (2.5%) who received the IRIV preparation. Severe pain was reported by one subject who received the alum-adsorbed vaccine. All other subjects who reported a "painful" reaction graded it as mild. Immunization with the alum-adsorbed vaccine was associated with a significantly ($P < 0.01$) higher incidence of both pain and swelling/induration compared with either the fluid or IRIV formulations. No systemic reactions attributable to vaccination were noted.

The anti-HAV antibody response engendered at various times postvaccination is shown in Table II. At 2 wk, immunization with the fluid vaccine yielded a geometric mean titer (GMT) of 16 mIU/ml with 30% of the subjects seroconverting (≥ 20 mIU/ml). Although the alum-adsorbed vaccine in-

Table I. Adverse Reactions Associated with Immunization

Vaccine	Local reactions (%)			Systemic reactions		
	Pain	Swelling/induration	Redness	Fever	Headache	Malaise
Fluid	42*	0‖	0	0	0	0
$Al(OH)_3$-adsorbed	88‡	23¶	0	0	0	0
IRIV	25§	5**	0	0	0	0

‡ versus * or §, $P < 0.01$. ¶ versus ‖ or **, $P < 0.01$.

duced both a moderately higher GMT (21 mIU/ml) and seroconversion rate (44%), neither was significantly greater than that obtained with the fluid vaccine. In contrast, the IRIV vaccine formulation elicited a far more vigorous antibody response. The GMT of 140 was significantly ($P < 0.0001$) higher compared with either of the two other vaccines. All but one vaccinee possessed ≥ 100 mIU/ml. Of greater importance was the fact that all vaccinees seroconverted by day 14 versus < 50% for the other vaccine formulations ($P < 0.005$).

By 4 wk, all subjects in the three groups had seroconverted and the anti-HAV antibody GMT had increased substantially. The GMTs obtained for the groups that received either the alum-adsorbed (871) or the IRIV formulation (831) were comparable, with both being significantly ($P < 0.001$) higher than that obtained with the fluid vaccine (388).

By 6 mo time, the GMTs for the groups receiving either the fluid or alum-adsorbed vaccines had declined by ~ 30% from the peak levels seen on day 28 and were not significantly different ($P > 0.05$). In contrast, the GMT of the IRIV vaccine recipients nearly doubled and was significantly ($P < 0.001$) higher than for the other two groups. The seroconversion rates were 80 and 95% for the recipients of the fluid and alum-adsorbed groups, respectively. In contrast, all subjects in the IRIV group still maintained > 20 mIU/ml with > 65% having > 1,000 mIU/ml.

By 1 yr postimmunization, fully 7 of 14 (50%) of the fluid vaccine group and 4 of 10 (40%) of the alum-adsorbed vaccine group possessed < 20 mIU/ml. All 22 subjects available for follow-up who had received the IRIV vaccine had > 20 mIU/ml, with six maintaining levels > 1,000 mIU/ml ($P < 0.01$). The GMT for the IRIV vaccine recipients was > 10-fold higher than for the other two vaccine groups.

Discussion

Currently, only aluminum-based salts are licensed as adjuvants for human use. Their adjuvant activity is due to their acting as a repository for adsorbed antigens and their propensity to induce a localized inflammatory response accompanied by recruitment of immune cells. Alum-adsorbed vaccines tend to elicit a high rate of mild to moderate reactions at the injection site. Unfortunately, such adjuvants do not appear to be sufficiently potent to markedly enhance the immune response to weak antigens. In addition, the use of aluminum-based adjuvants has been questioned due to their potential to cause encephalopathies (25).

We have attempted to develop a vaccine delivery system with innate adjuvant effects. The components of the described IRIV were selected with regard to both safety and immunopotentiating effect. PC is a constituent of intravenous reconstitution fluids for use in malnutrition. PE-containing liposomes are capable of stimulating B cells to secrete antibody to delivered antigens in the absence of a T cell determinant (26). The free amino group of PE can serve as a site to which antigens can be covalently coupled. Furthermore, as relates specifically to the presently described HAV vaccine, HAV preferentially attaches to the PE-rich areas of host cell membranes (27). Influenza HA possesses several theoretical immunopotentiating attributes that are exploited by the described IRIV. The HA1 subunit binds to the sialic acid residues of eucaryotic cells, including macrophages and lymphocytes. This could trigger receptor-mediated endocytosis of the IRIV via cell membrane fusion such as that which occurs during natural influenza virus infection (28). The HA2 subunit mediates the fusion of viral (and possibly IRIV) membranes with endosomal membranes. These steps, we theorized, could result in the efficient delivery of antigen when tightly complexed to the IRIV, thereby facilitating a rapid immune response. Additionally, most adolescents and adults possess memory cells to influenza HA with a broad specificity. This may also serve to enhance the response to an IRIV vaccine formulation through recruitment of primed cells.

Hepatitis A is prevalent in developing areas of the world. Although the disease is usually asymptomatic or mild in chil-

Table II. Immunogenicity of Fluid, $Al(OH)_3$-adsorbed, and IRIV-adjuvanted Hepatitis A Vaccines

Vaccine formulation	Geometric mean titer (range)				
	Day 0	Day 14	Day 28	Day 180	Day 352
			mIU/ml		
Fluid	<10	16 (<10–100)[A]	388 (100–>1,000)[D]	211 (14–1,043)[G]	39 (<1–133)[J]
$Al(OH)_3$-adsorbed	<10	21 (10–100)[B]	871 (100–1,000)[E]	535 (18–1,758)[H]	57 (12–211)[K]
IRIV-adjuvanted	<10	140 (25–300)[C]	831 (100–>1,000)[F]	1,499 (130–3,819)[I]	655 (59–2,112)[L]

Subjects received a single dose of vaccine on day 0. C vs. A or B, $P < 0.0001$; E or F vs. D, $P < 0.001$; E vs. F, $P > 0.05$; I vs. G or H, $P < 0.001$; H vs. G, $P > 0.05$; L vs. J or K, $P < 0.001$; J vs. K, $P > 0.05$.

dren, it can cause significant morbidity and mortality in adults and is a major risk for travelers to endemic areas. To combat this problem, formalin-inactivated hepatitis A vaccines have been developed (1, 29, 30) and tested in humans. Seroconversion rates at 2 wk postimmunization were reported to be roughly 40%, whereas at 4 wk the rate ranged from 25 to 94% depending upon the vaccine dose (1, 29, 30). A second dose of vaccine administered at 4 wk resulted in seroconversion rates of ≥ 90%. It would be desirable, both for travelers and for mass vaccination campaigns in underdeveloped areas, to have a vaccine capable of rapidly engendering a high rate of seroconversion after a single dose. In an attempt to induce a rapid anti-HAV antibody response, we complexed inactivated HAV to IRIV and compared its safety and immunogenicity with that of fluid and alum-adsorbed HAV vaccines.

Within 14 d of receiving a single dose of IRIV–HAV vaccine, all subjects seroconverted, possessing ≥ 20 mIU/ml of anti-HAV antibody/ml. In comparison, 30 and 44% of vaccinees immunized with the fluid or the alum-adsorbed vaccines, respectively, seroconverted ($P < 0.001$). The 44% rate of seroconversion observed with the alum-adsorbed vaccine 14 d postimmunization was comparable to that previously reported with a different formalin-inactivated, alum-adsorbed HAV vaccine (35–39%) (30). With the exception of the 1-mo time point, the IRIV-adjuvanted vaccine was superior to the alum-adsorbed vaccine as concerns both GMT and the percentage of vaccinees maintaining ≥ 20 mIU/ml. Therefore, although the anti-HAV antibody titers for the individuals who received either the alum-adsorbed or fluid vaccines progressively declined between 4 wk and 1 yr postvaccination, the peak GMT for the IRIV formulation was attained at 6 mo. After 1 yr, all the subjects who were immunized with the IRIV formulation possessed ≥ 20 mIU/ml compared with 50–60% for the other two groups. It therefore appears that the IRIV vaccine induces both a more rapid and longer lived anti-HAV antibody response than the two other conventional vaccine formulations. The difference observed between the three vaccine formulations could not be attributed to either antigen content or differences in the HAV antigen used since all three were produced from a single bulk lot of inactivated HAV and possessed the same antigen content. In addition to being more immunogenic, the IRIV vaccine was better tolerated than alum-adsorbed vaccines as judged by the incidence and severity of local adverse reactions.

The above findings demonstrate the safety and immunogenicity of an IRIV-formulated vaccine in humans. We have also recently found that an IRIV vaccine composed of influenza HA and neuraminidase of various serotypes also possesses enhanced immunogenicity in humans when compared with classical whole-virus vaccines (Glück, R., unpublished observation). Furthermore, the covalent coupling of nonimmunogenic peptides to IRIV was found to engender a good antipeptide antibody response in animals (Glück, R., R. Mischler, S. Brantschen, M. Just, B. Althaus, and S. J. Cryz, Jr., unpublished observations). The IRIV vaccine delivery system herein described may prove useful for the development of human vaccines containing a variety of antigens.

References

1. Flehmig, B., U. Heinrich, and M. Pfisterer. 1989. Immunogenicity of a killed hepatitis A vaccine in seronegative volunteers. *Lancet.* i:1039–1041.

2. Miller, L. H., R. J. Howard, R. Carter, M. F. Good, V. Nussenzweig, and R. S. Nussenzweig. 1986. Research towards malaria vaccines. *Science (Wash. DC).* 234:1349–1356.

3. Bolognesi, D. P. 1989. Progress in vaccine against AIDS. *Science (Wash. DC).* 246:1233–1234.

4. Good, M. F., S. Kumar, and L. H. Miller. 1988. The real difficulties for malaria vaccine development: nonresponsiveness and antigenic variation. *Immunol. Today.* 9:351–355.

5. Ribi, E., J. T. Ulrich, and K. N. Masini. 1987. Immunopotentiating activities of monophosphyryl lipid A. *In* Progress in Leukocyte Biology. Vol. 6. J. S. Madje, editor. Alan R. Liss, New York. 101–112.

6. Arnon, R., M. Shapiro, and C. O. Jacob. 1983. Synthetic vaccines. *J. Immunol. Methods.* 61:261–273.

7. Zanetti, M., E. Serearz, and J. Salk. 1987. The immunology of new generation vaccines. *Immunol. Today.* 8:18–25.

8. Morein, B., B. Sundquist, S. Höglund, K. Dalsgaard, and A. Osterhaus. 1984. ISCOM, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature (Lond.).* 308:457–460.

9. Lowell, G. H., W. R. Ballou, L. F. Smith, R. A. Wirtz, W. D. Zollinger, and W. D. Hockmeyer. 1988. Proteosome-lipopeptide vaccines: enhancement of immunogenicity for malaria CS peptides. *Science (Wash. DC).* 240:800–802.

10. Allison, A. C., and G. Gregoriadis. 1974. Liposomes as immunological adjuvants. *Nature (Lond.).* 252:252–255.

11. Garcon, N. M., and H. R. Six. 1991. Universal vaccine carrier. *J. Immunol.* 146:3697–3702.

12. Johnson, A. G., M. Toma, L. Solem, L. Beck, and E. Ribi. 1987. Characterization of nontoxic monophosphoral lipid A. *Rev. Infect. Dis.* 9:512–524.

13. Klausner, A. 1988. Adjuvants, a real shot in the arm for recombinant vaccines. *Biotechnology.* 6:773–777.

14. Staruch, M. J., and D. D. Wood. 1983. The adjuvanticity of interleukin 1 in vivo. *J. Immunol.* 130:2191–2196.

15. Morein, B., and K. Simons. 1985. Subunit vaccines against enveloped viruses: virosomes, micelles and other protein complexes. *Vaccine.* 3:83–93.

16. Perrin, P., L. Thibodeau, and P. Sureau. 1985. Rabies immunosome (subunit vaccine) structure and immunogenicity. Pre- and post-exposure studies. *Vaccine.* 3:325–332.

17. Watari, E., B. Dietzschold, G. Szokan, and E. Heber-Katz. 1987. A synthetic peptide induces long-term protection from lethal infection with herpes simplex virus 2. *J. Exp. Med.* 165:459–470.

18. Wiley, D. C., and J. J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 56:365–394.

19. Wharton, S. A., S. R. Martin, R. W. H. Ruigrok, J. J. Skehel, and D. C. Wiley. 1988. Membrane fusion peptide analogues of influenza virus haemagglutinin. *J. Gen. Virol.* 69:1847–1857.

20. Jackson, D. C., B. S. Crabb, P. Pombourious, W. R. Tulip, and W. G. Laver. 1991. Three antibody molecules can bind simultaneously to each monomer of the tetramer of influenza virus neuraminidase and the trimer of influenza virus hemagglutinin. *Arch. Virol.* 116:45–56.

21. Taylor, A. H., A. M. Haberman, W. Gerhard, and A. J. Caton. 1990. Structure-function relationships among highly diverse T cells that recognize a determinant from influenza virus hemaglutinin. *J. Exp. Med.* 172:1643–1651.

22. Flehmig, B., M. Ranhi, H. Frank, and H. J. Gerth. 1978. Application of a solid-phase radioimmunoassay and immune electron microscopy for hepatitis A in diagnosis and research. *Med. Microbiol. Immunol.* 166:187–194.

23. European Council (Strasbourg), editors. 1980. European Pharmacopoeia. 2nd ed Maisonneuve, Sainte-Ruffine, France.

24. Schild, G. C., M. Henry-Aymard, and H. G. Pereira. 1972. A quantitative, single-radial-diffusion test for immunological studies with influenza virus. *J. Gen. Virol.* 16:231–236.

25. Milliner, D. S., J. H. Shinaberger, P. Shuman, and J. W. Coburn. 1985. Inadvertent aluminum administration during plasma exchange due to aluminum contamination of albumin-replacement solutions. *N. Engl. J. Med.* 312:165–167.

26. Yasuda, T., G. F. Dancey, and S. C. Kinsky. 1977. Immunogenicity of liposome model membranes in mice: dependence on the phospholipid composition. *Proc. Natl. Acad. Sci. USA.* 74:1234–1239.

27. Seganti, L., F. Superti, N. Orsini, R. Gabrielli, M. Divizia, and A. Panà. 1989. Membrane lipid components interacting with hepatitis A virus. *Microbiologica (Pavia).* 12:225–230.

28. Matlin, K. S., H. Reggio, A. Helenius, and K. Simons. 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. *J. Cell Biol.* 19:601–613.

29. Wiedermann, G., F. Ambrosch, H. Kollaritch, H. Hofmann, C. Kunz, E. D'Hondt, A. Delem, F. E. André, A. Safary, and J. Stéphenne. 1990. Safety and immunogenicity of an inactivated hepatitis A candidate vaccine in healthy adult volunteers. *Vaccine.* 8:581–584.

30. Hess, G., B. May, H. Chriske, M. Dietrich, H. Bock, and the HAV Collaborative Study Group. 1990. Safety and immunogenicity of an inactivated hepatitis A virus (HAV) in two adult age groups. *Intersci. Conf. Antimicrobiol. Agents Chemother.* 30:147. (Abstr. 361)