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Anti-Idiotypic Antibody Vaccine for Type B Viral Hepatitis in Chimpanzees

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Anti-idiotypic antibodies (anti-Id) that contain an internal image component that mimics the surface antigen of hepatitis B virus (HBsAg) were used to immunize chimpanzees. Four injections of the rabbit anti-Id preparation elicited an antibody response to HBsAg (anti-HBs). The antibody specificity appeared to be against the anti-Id, since the anti-Id immunogen was shown to bind the chimpanzee anti-HBs. Two chimpanzees immunized with the anti-Id, along with two control animals that were either untreated or received a nonimmune rabbit immunoglobulin G preparation, were challenged with infectious hepatitis B virus. Both control chimpanzees developed clinical and serological characteristics consistent with an active hepatitis B virus infection, whereas the two anti-Id treated chimpanzees were protected from infection. Since chimpanzees provide a relevant model of a human response to hepatitis B virus immunization and infection, these results indicate that anti-Id preparations such as that described here might be candidates for vaccines against human diseases.

A SUBUNIT VACCINE FOR HEPATITIS B virus (HBV) is now available and is produced by purifying HBV surface antigen (HBsAg) from the plasma of persons chronically infected with HBV. Although this vaccine is safe and effective, its high cost is likely to preclude its use in developing countries where HBV and its sequelae constitute a major health problem. Several alternative approaches to the preparation of HBsAg vaccines are under investigation. In one, DNA fragments of HBV are cloned into suitable vectors (*1, 2*). Studies in chimpanzees have demonstrated that this recombinant HBsAg approach is a possible means for a vaccine production (*3–5*). In another approach, synthetic peptides that contain amino acid sequences analogous to those associated with the major protein component of HBsAg are used (*6*). However, these synthetic HBsAg peptides may only partially protect chimpanzees from challenge with infectious HBV (*7*). In a third approach, being studied at our laboratories, an anti-idiotypic antibody (anti-Id) preparation that represents the internal image of HBsAg is used as the vaccine.

An idiotype or idiotypic determinant (Id) defines the variable (V) region of an antibody molecule. Initial studies by Kunkel *et al.* (*8*) and Oudin and Michael (*9*) characterized these antigenic determinants on human and rabbit antibodies, respectively, by generating anti-Id reagents. Id, along with homologous anti-Id, are thought to be components of a network of complex reactions that regulates a given immune response. Initially proposed by Jerne (*10*), Id networks have been implicated in regulating the immune response to a wide variety of haptens and protein or carbohydrate antigens (*11, 12*). More recently, anti-Id have been used to induce an immune response to antigens associated with a large number of pathogens (*13*). Previous studies at our laboratories (*14*) led to the characterization of a common Id on human antibodies to HBsAg. The anti-Id produced in rabbits against the human Id antibody to HBsAg appeared to contain an internal image component that mimicked HBsAg: This was deduced from (i) the detection of an interspecies Id cross-reaction associated with antibodies to HBsAg (*15*); and (ii) the production in mice of antibodies to HBsAg that serologically resembled the human Id antibody to HBsAg produced by injecting anti-Id alone (*16, 17*). Together these data indicated that this internal image anti-Id might be useful as a vaccine against HBV.

In the present studies, human antibodies to HBsAg served as the idiotype or first antibody (Ab-1) that was injected into rabbits to produce the anti-Id or second antibody (Ab-2). The rabbit anti-Id or Ab-2 was tested for its vaccine potential. Two chimpanzees that received four injections of affinity-purified rabbit anti-Id developed a detectable anti-HBs response 1 week (week 12) after the final immunization (Fig. 1). Although 1 mg of anti-Id per injection represents a large dose when compared to conventional vaccines, it is noteworthy that the anti-Id is a polyclonal antibody preparation and only a percentage of the total anti-Id may represent the internal image. Presumably an internal image anti-Id that was produced monoclonally might contain a larger proportion of internal image anti-Id so that a lower dose of vaccine could be used for vaccination. One chimpanzee (X-33) that was injected with nonimmune rabbit immunoglobulin G (IgG) on a similar schedule did not develop an anti-HBs response before being challenged with infectious HBV. In addition, an untreated control chimpanzee (X-188), seronegative for anti-HBs, was included in this study and showed no antibody response to HBV infection. These data suggested that the anti-Id immunization was responsible for inducing anti-HBs.

To ascertain whether the chimpanzee anti–HBs was associated with an anti-Id response to the rabbit anti-Id (a chimpanzee anti–anti-Id response), serum containing anti-HBs was obtained from each chimpanzee before it was challenged with virus (week 13 to 23). This serum was pooled and repeatedly adsorbed over an immunoadsorbent column coated with nonimmune rabbit IgG. The presence of anti-isotypic and anti-allotypic activity along with anti-idiotypic activity against the rabbit anti-Id was determined by a direct binding radioimmunoassay (RIA). An isotype is an antigen that determines the class and subclass differences found on immunoglobulin molecules within

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a given species. Immunoglobulins M and G are two common isotypes that can be detected. Allotypes are intraspecies polymorphisms found on antibody molecules. An allotype is to an antibody what ABO blood groups are to red blood cells. As shown in Fig. 2, the two adsorbed chimpanzee antisera bound only to the rabbit anti-Id immunogen and not to rabbit IgG from either a control, affinity-purified anti-Id preparation or a nonimmune pool of sera. The nonimmune pool contained approximately 50 percent IgG by weight from the preimmune serum of the anti-Id immunogen donor (rabbit). This nonimmune IgG preparation would be expected to share the same allotypic determinants as the anti-Id used for injection. The adsorbed chimpanzee antisera still possessed anti-HBs activity in titers comparable to the unadsorbed antisera. These data indicated that the anti-HBs response resulted from an anti-Id response to the rabbit anti-Id; namely, a chimpanzee anti–anti-Id (Ab-3) response.

We next determined whether the chimpanzee anti-Id–induced anti-HBs response would protect chimpanzees from infectious HBV challenge. As shown in Fig. 3, the two chimpanzees that received rabbit anti-Id, except for showing consistent anti-HBs titers, were negative for all serological markers of HBV infection after challenge with HBV. Serum concentrations of the liver enzyme alanine aminotransferase (ALT) remained constant in these two chimpanzees, which also indicated that the animals were protected. In contrast, the two control animals (X-33 and X-188) developed HBV infections and became positive for HBsAg and antibody to hepatitis B core antigen and showed increased concentrations of ALT. HBsAg was detected 4 weeks (week 28) and 5 weeks (week 29) after challenge with HBV in X-33 and X-188, respectively. Hepatitis B e antigen, associated with infectious Dane particles, was detected in both animals 7 weeks (week 31) after challenge, and

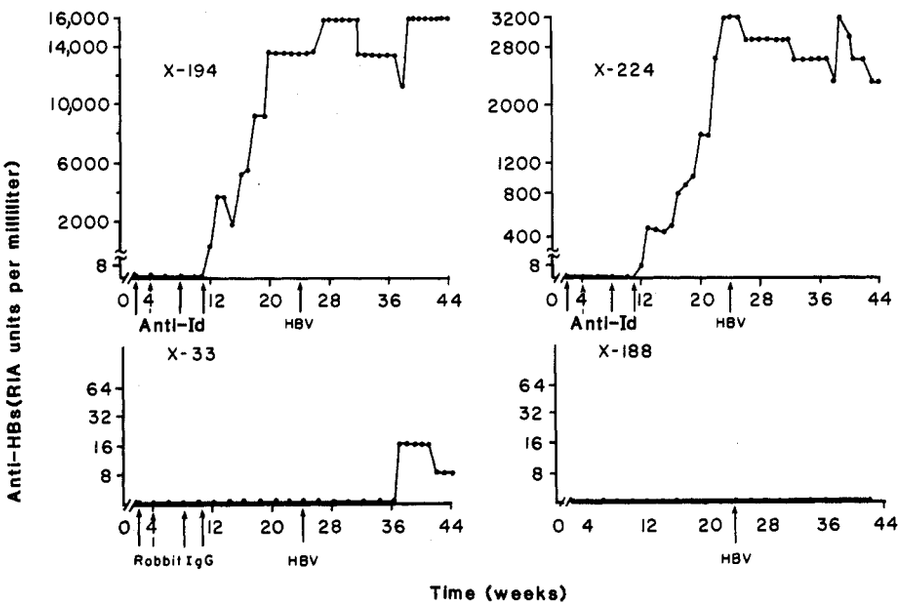

Fig. 1. Immunization schedule and subsequent antibody response to HBsAg in two vaccinated and two control chimpanzees. The anti-HBs response was assayed by a commercial RIA referred to as AUSAB (Abbott) and is expressed as RIA units per milliliter of serum as determined according to the manufacturer's specifications. Chimpanzees X-194 and X-224 were immunized four times each with 1 mg of affinity-purified rabbit anti-Id vaccine adsorbed to alum. This dose was selected on the basis of previous studies in mice where the minimum amount of anti-Id required to induce an anti-HBs was determined. The dose approximates a weight per weight basis of the chimpanzee relative to the mouse. Chimpanzee X-33 was inoculated with a similar concentration of nonimmune rabbit IgG preparation adsorbed to alum. Chimpanzee X-188 was untreated prior to HBV inoculation. All animals were challenged on week 24 with approximately 3000 chimpanzee infectious doses of HBV.

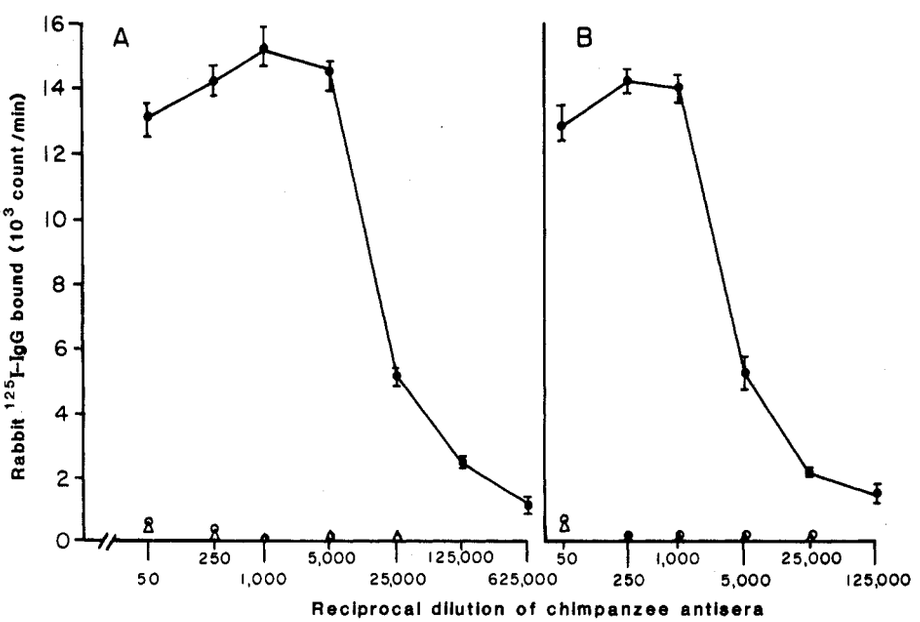

Fig. 2. Binding curves of chimpanzee anti–anti-Id with anti-Id immunogen (●), a control SV40 T-ag rabbit anti-Id (○) (*31*), and purified rabbit IgG from a pool of nonimmune sera (△). The following chimpanzee anti–anti-Id were used: (A) X-194; (B) X-224. Chimpanzee immune sera, positive for anti-HBs obtained prior to HBV challenge (weeks 13 to 23), were pooled and repeatedly adsorbed over a Sepharose 4B affinity column coupled with pooled nonimmune rabbit IgG until all detectable reactivity with isotypic and allotypic determinants was removed. The methods for generating heterologous anti-Id reagents and the preparation of affinity columns with the use of cyanogen bromide–actived Sepharose have been described previously (*32*). Anti-isotypic and anti-allotypic antibody activity in the adsorbed chimpanzee antisera was determined by a direct binding, solid phase radioimmunoassay (RIA). Briefly, chimpanzee sera diluted in borate-buffered saline was adsorbed to the wells of microtiter plates overnight at 4°C. After nonspecific sites were blocked with 10% normal goat serum (NGS), 50 μl of the suspension containing 100,000 cpm of ^{125}I-labeled rabbit IgG preparations was added. These preparations included anti-HBs anti-Id (immunogen), anti-SV40 T-ag anti-Id, and nonimmune rabbit IgG. All rabbit antibodies were iodinated by the chloramine-T method. After incubation for 18 hours at 4°C, the residual radioactivity was removed and the plates were washed with a Tween-20 phosphate-buffered saline (*15*). The wells of the microtiter plates were cut out, and bound radioactivity was counted in an automatic gamma counter. The binding curves are expressed as counts per minute of ^{125}I-labeled rabbit IgG bound versus dilution of chimpanzee antisera. Each point represents the mean of triplicate values and the brackets refer to the range of values.

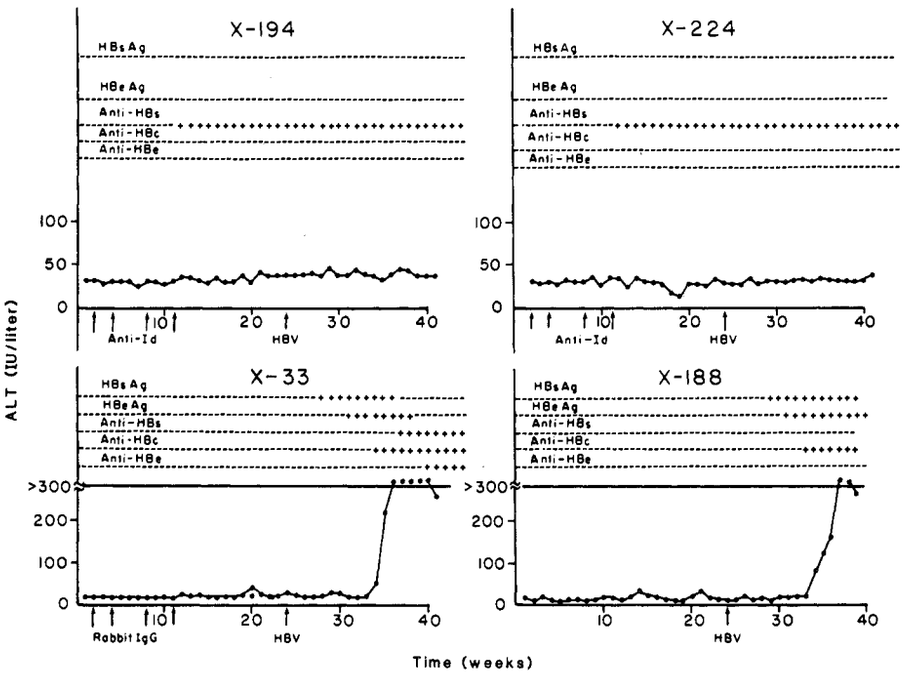

Fig. 3. An enzymatic and serological profile of serum samples taken from the four chimpanzees; two that received anti-Id vaccine and two controls. Serum alanine aminotransferase (ALT) was determined by a commercial Worthington kit (Cooper Biomedical) and is expressed as international units per liter. HBsAg, HBeAg, anti-HBs, anti-HBc, and anti-HBe were assayed by commercial RIA kits (Abbott). The serological profile is expressed as positive (+) or negative (−) for the various HBV markers according to the manufacturer's specifications. Liver biopsies were obtained with a 16-gauge Klatskin liver biopsy needle at monthly intervals. The biopsy material was placed in 10% buffered Formalin and examined by a veterinary pathologist.

subsequently there was an anti-HBe response. Concentrations of ALT peaked at 904 and 399 IU per liter for X-33 and X-188, respectively.

The vaccine study was continued for a total of 70 weeks. Chimpanzee X-188 developed an anti-HBs response at week 48. No serological markers of an HBV infection were detected in the two anti-Id treated chimpanzees during the entire course of this study. Normal liver histology confirmed that the two anti-Id treated chimpanzees were protected from the HBV challenge. To determine whether HBV replication occurred in the anti-Id treated chimpanzees, we analyzed serum samples by dot blot hybridization for the presence of HBV DNA. Serum samples (5, 50, and 250 μl) were applied to nitrocellulose with the use of a microvacuum manifold. The nitrocellulose was treated sequentially with 0.5*N* NaOH–1.5*M* NaCl; 1*M* tris (*p*H 7.4)–1.5*M* NaCl, and 0.5*M* tris (*p*H 7.4)–1.5*M* NaCl. The filter was baked at 80°C for 2 hours and hybridized at 65°C for 16 hours with a ^{32}P-labeled nick-translated probe. The probe was a genomic copy of HBV DNA cloned into the Eco RI site of pUC18. Unlabeled denatured HBV plasmid was applied to the nitrocellulose at concentrations from 0.1 μg to 0.1 pg to quantitate the level of hybridization observed from the serum samples. In addition, human serum samples positive for HBsAg (5 and 50 μl) were applied to the filter to demonstrate the sensitivity of the assay for virion-associated DNA in serum. The plasmid quantitation demonstrated that the hybridization technique was capable of detecting 0.4 pg of DNA, and examination of human serum samples revealed that HBV DNA was detectable in as little as 5 μl of serum. Analyses of the sera from anti-Id immunized chimpanzees indicated that HBV DNA was either absent or present at a concentration of less than 0.4 pg per milliliter of serum. These data suggest that HBV replication did not occur at detectable levels in the vaccinated chimpanzees. Together these data indicate the efficacy of an anti-Id vaccine for type B viral hepatitis.

The theoretical implications of using anti-Id preparations as vaccines have been discussed previously (*18, 19*). The induction of an immune response or protective immunity has been demonstrated in murine systems for trypanosomiasis (*20*), rabies virus (*21*), reovirus (*22*), Sendai virus (*23*), pneumococcus (*24*), *Escherichia coli* polysaccharide capsule (*25*), poliovirus (*26*), *Listeria* (*27*), and herpes simplex virus type 2 (*28*), as well as HBV (*16*). Recently, an anti-Id preparation was used to induce partial protective immunity (50 to 80 percent) in rats subsequently infected with *Schistosoma mansoni* (*29*). However, the rat is a nonpermissive host in the context of schistosomiasis and therefore may not represent an adequate experimental animal model for extrapolation of the results to humans. Data from the studies cited above suggested that the use of anti-Id vaccines might be limited. For example, some data indicated that the anti-Id protection against *Trypanosoma* was related only to certain inbred strains of mice (*30*). Such a vaccine would have little value in an outbred population such as humans. The potential problem with the anti-Id vaccine for *Trypanosoma* was that it did not appear to contain an internal image population. Internal image anti-Id vaccines should be no more restricted than the antigen they mimic. Indeed, the internal image anti-Id that mimics HBsAg induces an anti-HBs response in BALB/c mice, rabbits, and chimpanzees.

Another potential question about anti-Id vaccines is how long the immunity will last. We selected 13 weeks after the last injection to challenge the chimpanzees because the anti-HBs titers were relatively constant (Fig. 1). However, if one examines the anti-HBs response upon challenge with HBV in the control chimpanzees and compares this with the relatively consistent anti-HBs titers from weeks 24 to 36 of the vaccinated animals, challenge with infectious HBV could have taken place 25 weeks after the final injection (week 36) and protection would have still been reasonably assured. Anti-HBs titers in the two vaccinated chimpanzees were constant 11 months after challenge (week 70). Recent antibody titers were 13200 and 2820 RIA units/ml for chimpanzee X-194 and X-224, respectively. Thus, anti-Id induced immunity, at least for HBV, does not appear to be transient. Because of the expense of chimpanzees, two vaccinated and two control animals are considered reasonable for testing the safety and efficacy of an HBV vaccine candidate (*4, 7*). There may also be some concern about multiple injections of a heterologous antibody preparation and the potential for inducing an anaphylactic reaction. In our experiments, three chimpanzees received a total of 4 mg of rabbit IgG (two vaccine and one control) in four injections and showed no adverse reactions. Serum fractionation procedures indicated that the antibody response to rabbit IgG in the chimpanzees was IgG in nature. Skin testing of the treated chimpanzees (PK reaction) with the alum-precipitated rabbit anti-Id immunogen did not demonstrate any homocytotropic antibodies that could mediate an immediate-type allergic reaction.

A practical application of anti-Id vaccines for the future may be the protection of infant humans and other animals against

pathogenic capsule-forming bacteria such as the pneumococcus (*24, 25*). Newborns do not develop the ability to respond to the capsular polysaccharide of these bacteria until late in ontogeny and are thus extremely susceptible to such pathogens. Administration of an anti-Id vaccine early in life could prime the immune system to produce protective antibodies upon subsequent antigen exposure (*25*). Anti-Id vaccines might also be useful for adults who do not respond to conventional vaccines, such as the small percentage of individuals vaccinated with the current HBV vaccine who fail to produce anti-HBs. The studies reported herein demonstrate the efficacy of such a vaccine for HBV in chimpanzees, and suggest that internal image anti-Id may be candidates for developing vaccines against other infectious agents.

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Primary Rat Embryo Cells Transformed by One or Two Oncogenes Show Different Metastatic Potentials

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Second-passage rat embryo cells were transfected with a neomycin resistance gene and the activated form of the c-Ha-*ras* I gene, or with these two genes plus the adenovirus type 2 E1a gene. Foci of morphologically transformed cells were observed in both cases; however, the frequency of transformation was at least ten times higher with two oncogenes than with the *ras* gene alone. All the transformed cell lines gave rise to rapidly growing tumors when injected subcutaneously into nude mice. All but one of the cell lines transformed by the *ras* oncogene alone formed metastatic nodules in the lungs of animals that had been injected subcutaneously with transformed cells. When transformed cells were injected intravenously, all the *ras* single-gene transformants gave rise to many metastatic lung nodules. In contrast, cell lines transformed with *ras* and E1a did not generate metastases after subcutaneous injection and gave rise to very few metastatic lung nodules after intravenous injection. These data demonstrate that a fully malignant cell with metastatic potential, as measured in an immunodeficient animal, can be obtained from early passage embryo cells by the transfection of the *ras* oncogene alone.

DNA TRANSFECTION EXPERIMENTS have identified dominant transforming genes from the genomes of tumor cell lines and tumor tissues (*1–3*). Studies on the early region transforming genes of polyoma virus and adenovirus indicated that two viral genes are required to stably transform primary rodent cells in culture (*4–7*). The role of cellular oncogenes in the transformation of primary rodent cells has been examined by two groups of independent investigators (*8, 9*), who showed that two so-called cooperating oncogenes are required to stably transform primary cells in culture. Later, the transformation of primary cells with a single oncogene was described (*10, 11*).

We now report the transformation of second-passage rat embryo cells after transfection of the single oncogene c-Ha-*ras*. The frequency of focus formation was found to be ten times greater when a second oncogene, the adenovirus E1a gene, was cotransfected with c-*ras*. We examined the phenotypic differences between cell lines transformed by one or two oncogenes. The most striking difference between the two types of transformants is that cells transformed by the c-*ras* oncogene alone exhibited a greater propensity to form metastatic lesions after injection into nude mice than did cells transformed by two oncogenes.

To study the effects of the *ras* oncogene on early passage rat embryo cells, we constructed the plasmids pSV2ras, pRasEn1, and pRasEn2 (Fig. 1); pSV2ras contains the activated form of the c-Ha-*ras*-I gene, as cloned from the bladder carcinoma cell line T24 (*12*), under the transcriptional control of the SV40 early region promoter-enhancer element. We constructed the plasmid pRasEn1 by inserting a 234-bp DNA fragment containing the SV40 72-bp and 21-bp repeat elements into pEJ at a position roughly 2.5 kb distal to the putative polyadenylation signal of the *ras* gene (*13*). The plasmid

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