Antibodies to hepatitis A virus (anti-HAV) were measured in children from two separate vaccine trials (n = 70) 4 weeks after a dose of inactivated hepatitis A vaccine (VAQTA). The geometric mean titers (GMTs) of anti-HAV were 49.3 and 45.2 mIU/mL by immunoassay, while reciprocal GMTs of neutralizing anti-HAV were 6.5 and 15.0 by an 80% radioimmunofocus inhibition test (RIFIT) and 55.6 and 92.0 by antigen reduction assay (HAVARNA). The GMT of antibody detected by radioimmunoprecipitation (RIPA) was \( \approx 401 \). These data establish serologic correlates of protection against disease and show that RIPA is most sensitive for detection of early vaccine-induced antibody. Sera collected from adults (n = 20) 7 days after administration of immune globulin contained similar antibody levels by immunoassay (45.1 mIU/mL) and slightly higher GMTs of neutralizing antibody (27.5 by RIFIT and 146 by HAVARNA) but negligible precipitating antibody (GMT, 5.6). These results are best explained by differences in the affinity of antibodies for virus following active versus passive immunization.

Inactivated hepatitis A virus (HAV) vaccines are safe and highly effective in prevention of symptomatic hepatitis A in immunized children, and they offer substantial promise for control of this disease [1–3]. Although the immunologic correlates of protection are not well-defined, the well-documented ability of passively transferred antibodies to protect against hepatitis A suggests that the protection afforded by active immunization with formalin-killed virus vaccines is due principally if not entirely to the production of circulating antibodies capable of neutralizing HAV [4, 5]. Passive immunization with immune globulin (IG) at usual doses (0.02–0.06 mL/kg of body weight) results in only low levels of such antibodies and generally does not lead to detectable seroconversion when paired serum samples are tested by commercially available competitive inhibition immunoadsorbents for anti-HAV antibodies (e.g., HAVAB; Abbott, Abbott Park, IL) [6, 7]. While these low levels of serum anti-HAV antibodies provide a relatively high degree of clinical protection against disease, the minimal protective level of anti-HAV is not known.

In a controlled clinical trial, a formalin-inactivated hepatitis A vaccine (VAQTA; Merck Research Laboratories, West Point, PA) [8, 9] induced complete protection against clinically apparent hepatitis A (100% observed efficacy; confidence interval [CI], 97%–100%) by 30 days following administration of a single 25–antigen unit (U) dose to children aged 2–16 years [2]. Here we characterize the protective antibody response present 4 weeks after such immunization. Antibodies to HAV were measured by conventional solid-phase immunoassay (modified HAVAB) in comparison to an international reference reagent, by two different assays for HAV-neutralizing antibodies, the radioimmunofocus inhibition test (RIFIT) [10] and HAV antigen reduction assay (HAVARNA) [11], and by a novel radioimmunoprecipitation assay (RIPA) for detection of anti-HAV [12]. We describe protective levels of vaccine-induced anti-HAV antibodies as defined by each of these assay methods and compare these results with those obtained in a cohort of adults who had received passive immunization with IG.

Materials and Methods

Study subjects. Serum specimens were collected from children enrolled in two separate clinical trials of a recently licensed inactivated HAV vaccine (VAQTA). MRL-020 was an open-label study of the immunogenicity and tolerability of vaccine administered to children 4–12 years of age. A subset of 50 children (mean age, 8.9 years) who had been enrolled in this trial and who had received a single 25-U dose of vaccine was randomly selected from one of the study sites. Sera that had been collected from these children prior to and 4 weeks after administration of vaccine were studied for the presence of antibody. MRL-023 was a large, randomized, double-blind, placebo-controlled clinical trial that demonstrated the efficacy of a single 25-U dose of this vaccine in prevention of clinically evident hepatitis A in children aged 2–16 years [2].
demonstration of efficacy, a subset of enrolled children received a 25-U booster dose of vaccine ~24 weeks after their primary dose. A subset of 20 immunized children (mean age, 7.8 years) who had not experienced clinical signs or symptoms of hepatitis A was selected randomly, and sera collected prior to and 4 weeks after both primary (weeks 0 and 4) and booster (weeks 24 and 28) immunizations were tested for anti-HAV activity. These results were compared with antibody levels in 20 healthy adult subjects (mean age, 27.3 years) 7 days after administration of a single intramuscular injection of IG (0.06 mL/kg) (MRL-005).

**Modified HAVAB RIA for anti-HAV.** Antibody to HAV was measured by a modification of the commercial HAVAB RIA (Abbott). This modified HAVAB was carried out by mixing 100 µL of serum with 100 µL of 125I-labeled anti-HAV, instead of mixing 10 µL of serum and 200 µL of 125I-labeled anti-HAV as directed by the manufacturer [13, 14]. Quantitation of antibody activity was based on a standard curve constructed from a serial 2-fold dilution of the World Health Organization’s reference immunoglobulin (5–320 mIU/mL) [15]. All samples and standards were diluted in normal human serum as required. Extensive testing has demonstrated that levels of antibody as low as 5 mIU/mL can be reliably detected by this method [14]. However, the counts per minute (cpm) value obtained from the 10 mIU/mL point on the standard curve was selected as a more conservative break point for positivity in the modified HAVAB.

**RIFIT for neutralizing antibody to HAV.** This assay for serum neutralizing antibody is based on the ability of anti-HAV to inhibit the development of macroscopic foci of viral replication in cells overlaid with agarose [10, 16]. RIFIT was carried out as described previously, with the following modifications. Serial dilutions of serum (1:2, 1:8, 1:32, and 1:128 [MRL-020], or 1:8, 1:80, and 1:800 [MRL-023 and MRL-005]) were mixed with equal volumes of the virus suspension (HM175/18f virus) prior to inoculation onto nearly confluent cultures of BS-C-1 cells. Radioimmunofoci were enumerated after 6 or 7 days of growth. Serum samples were considered positive for neutralizing antibodies if a 1:8 initial dilution of serum inhibited the development of ≥80% of the radioimmunofoci developing in the absence of human serum. Neutralizing antibody titer was estimated from the calculated 80% radioimmunofocus reduction end point determined by regression analysis over the range of serum dilutions tested. The titer reported for most of the 4-week-postimmunization sera (58 of 70 samples) represents the geometric mean titer (GMT) of results obtained in two or three separate assays. Other reported titers represent the results of single assays.

**HAVARNA.** HAVARNAs were carried out by mixing 100 µL of serum with 100 µL of 125I-labeled anti-HAV, instead of mixing 10 µL of serum and 200 µL of 125I-labeled anti-HAV as directed by the manufacturer [13, 14]. Quantitation of antibody activity was based on a standard curve constructed from a serial 2-fold dilution of the World Health Organization’s reference immunoglobulin (5–320 mIU/mL) [15]. All samples and standards were diluted in normal human serum as required. Extensive testing has demonstrated that levels of antibody as low as 5 mIU/mL can be reliably detected by this method [14]. However, the counts per minute (cpm) value obtained from the 10 mIU/mL point on the standard curve was selected as a more conservative break point for positivity in the modified HAVAB.

**Neutralizing antibody response to inactivated HAV vaccine.** Sera collected from 70 children at week 4 after a single dose of inactivated HAV vaccine (VAQTA) were tested for HAV-specific antibody activity. These children were participants in two separate clinical studies, MRL-020 (a vaccine immunogenicity study) and MRL-023 (the Monroe, NY, efficacy study) [2]. All of the participants in these studies were prescreened for anti-HAV antibody, and only seronegative children (≤10 mIU/mL) were enrolled. Four weeks after immunization, the GMTs of anti-HAV antibodies detected by modified HAVAB [13, 14] in participants in the two studies were 49.3 and 45.2 mIU/mL, respectively (table 1).

Neutralizing antibodies were measured by both the RIFIT [10] and the HAVARNA [11], and the results of these assays were compared with each other and with the results of the modified HAVAB assay (table 1). Thirty-nine (56%) of 70 sera contained neutralizing antibodies detectable by RIFIT (≥80% reduction in replication focus number at a 1:8 serum dilution), while 56 (98%) of 57 tested sera contained neutralizing antibodies detectable in the HAVARNA (≥50% antigen reduction at a 1:4 serum dilution). Although the HAVARNA appeared to be more sensitive than the RIFIT for detection of the early neutralizing antibody response to HAV vaccine, it is important to note that the RIFIT imposes a more stringent criterion for positivity. This is reflected in differences in the GMT of neutralizing antibodies detected by these two methods. By RIFIT, the reciprocal GMT for neutralizing antibody was 6.5 and 15.0 for the MRL-020 and MRL-023 vaccine recipients, respectively, compared with 55.6 and 92.0 by HAVARNA (table 1). Thus, although antibody responses in the two studies were similar when assessed by the modified HAVAB assay (P =
Table 1. Measurement of antibody to HAV in adult recipients of immune globulin (7 days) and pediatric subjects (4 weeks) after single dose of inactivated vaccine.

<table>
<thead>
<tr>
<th>Assay</th>
<th>MRL-020 vaccine study</th>
<th>MRL-023 vaccine study</th>
<th>MRL-005 immune globulin study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive/total (%)</td>
<td>GMT (95% CI)</td>
<td>No. positive/total (%)</td>
</tr>
<tr>
<td>Modified immunoassay (HAVAB, mIU/mL)*</td>
<td>50/50 (100)</td>
<td>49.3 (43–57)</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>Radioimmunofocus inhibition (RIFIT) (80% inhibition at 1:8 serum dilution)</td>
<td>22/50 (44)</td>
<td>6.5 (5–8)</td>
<td>17/20 (85)</td>
</tr>
<tr>
<td>HAV antigen reduction (HAVARNA) (50% inhibition at 1:4 serum dilution)</td>
<td>36/37 (97)</td>
<td>55.6 (38–81)</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>3H-labeled HAV immunoprecipitation (RIPA) (30% precipitation at 1:8 or 1:80 dilution)</td>
<td>42/43 (98)</td>
<td>ND</td>
<td>20/20 (100)</td>
</tr>
</tbody>
</table>

NOTE: For calculation of geometric mean titers (GMTs): RIFIT, <1:8 = 4; HAVARNA, <1:4 = 2; RIPA, <1:8 = 4, ≥800 = 800. CI, confidence interval. ND, not determined.

* Positive considered ≥10 mIU/mL.

Table 2. Measurement of antibody to HAV in MRL-023 study subjects at 4, 24, and 28 weeks.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Week 4 (mean, 35.6 days)</th>
<th>Week 24 (mean, 218 days)</th>
<th>Week 28 (mean, 254 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive/total (%)</td>
<td>GMT (95% CI)</td>
<td>No. positive/total (%)</td>
</tr>
<tr>
<td>Modified immunoassay (HAVAB, mIU/mL)*</td>
<td>20/20 (100)</td>
<td>45.2 (37–55)</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>Radioimmunofocus inhibition (RIFIT)</td>
<td>17/20 (85)</td>
<td>15.0 (10–24)</td>
<td>12/20 (60)</td>
</tr>
<tr>
<td>HAV antigen reduction (HAVARNA)</td>
<td>20/20 (100)</td>
<td>92.0 (55–154)</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>3H-labeled HAV immunoprecipitation (RIPA)</td>
<td>20/20 (100)</td>
<td>≥401 (242 to ≥665)</td>
<td>19/20 (95)</td>
</tr>
</tbody>
</table>

NOTE: For calculation of geometric mean titers (GMTs): RIFIT, <1:8 = 4, ≥1:800 = 800; HAVARNA, <1:4 = 2; RIPA, <1:8 = 4, ≥800 = 800. CI, confidence interval.

* Between weeks 24 and 28.

† Positive considered ≥10 mIU/mL.
A comparison of RIFIT and HAVARNA titers in individual serum samples revealed only a weak correlation when only 4- and 24-week sera were examined in a combined analysis of the MRL-020 and MRL-023 studies (figure 1). This low level of correlation may have been due to a combination of factors, including interassay variability, the discrete nature of the neutralization assay measurements, and the narrow range of response within each sampling interval. The interassay variability appeared to be less with RIFIT than with HAVARNA. In replicate assays, only 1 of 47 paired results obtained by RIFIT differed by a >4-fold factor, while this was the case with 8 of 36 replicate HAVARNA results (data not shown). A stronger correlation between RIFIT and HAVARNA was evident when sera collected from MRL-020 study participants at week 28 (following booster immunization) were included in the analysis (figure 1). Similarly, titers obtained in either virus neutralization assay correlated with the antibody content of serum as determined by quantitative modified HAVAB (figure 2). Both neutralization assays were able to detect antibody concentrations below 100 mIU/mL (the approximate detection limit for the standard HAVAB test), although many such sera were negative by RIFIT. As shown in figure 2 (top), all of the postimmunization serum samples that were negative by RIFIT (titer <1:8) contained <100 mIU of antibody/mL by modified HAVAB.

Detection of vaccine-induced antibodies by immunoprecipitation of \( ^{3}H \)-labeled virus. To assess immunoprecipitation as a means of monitoring the response to immunization with inactivated HAV vaccine, virus was radiolabeled by growth in the presence of \( ^{3}H \)uridine and partially purified for use as antigen in RIPA [12]. The specific activity of this virus was \( \sim 300 \) cpm/10\(^{6}\) radioimmunofocus-forming units [18], at least 80% of which could be precipitated by appropriate dilutions of anti-HAV-positive serum samples (data not shown).

Paired serum samples from children enrolled in MRL-020, collected at weeks 0 and 4 after immunization, were tested for anti-HAV activity by RIPA at 1:8 and 1:80 serum dilutions. A representative sample of these results, showing the percentage of radiolabel precipitated by paired serum samples at both dilutions, is depicted in figure 3. With most preimmunization serum samples, less virus was precipitated at the 1:80 dilution than at the 1:8 dilution, reflecting less nonspecific precipitation of the virus at the higher serum dilution. In contrast, dilution had an opposite effect with most sera collected after immunization. Generally higher precipitation values were obtained at 1:80 compared with 1:8 serum dilutions, indicating a prozone effect with many of these postimmunization sera (figure 3). To establish criteria for positivity in this RIPA, an extended panel of paired sera from the MRL-020 study was tested at both 1:8 and 1:80 dilutions. When preimmunization sera (\( n = 40 \)) were tested at a 1:8 dilution, the mean (+SD) percentage of label precipitated was 7.98% ± 4.66%. At a 1:80 dilution, the mean percentage of label precipitated with the preimmunization sera (\( n = 38 \)) was 5.98% ± 4.08%. Comparable results with paired serum samples collected 4 weeks after immunization were 43.0% ± 19.3% (1:8 dilution) and 66.0% ± 16.4% SD (1:80 dilution). On the basis of these results, serum samples capable of precipitating \( \geq 30\% \) of label at either 1:8 or 1:80 serum dilutions were considered positive for HAV-specific precipitating antibodies. By application of this conservative criterion, each of the postimmunization sera was negative by RIPA, while 37 of 38 postimmunization sera were positive, 26 (68%) at a 1:8 dilution and 37 (97%) at a 1:80 dilution (data not shown).

Similar results were obtained when serum samples collected at weeks 0, 4, 24, and 28 from the MRL-023 study subjects were tested for the presence of precipitating antibodies. The postimmunization sera were tested at 1:8, 1:80, and 1:800 dilutions. At the 1:8 dilution, 11 (55%) of 20 serum samples collected 4 weeks after immunization and 18 (90%) of 20 samples collected 24 weeks after immunization were positive for antibody (\( \geq 30\% \) precipitation) (figure 4B, C). The reduced prozone effect observed with the 24-week sera correlated with a
slight increase in antibody content between weeks 4 and 24 suggested by modified HAVAB (GMT, 45.2 vs. 52.9) and slightly lower GMTs determined by both RIFIT and HAVARNA at 24 weeks (table 1). There was substantial variation in the percentage of virus precipitated by paired sera collected from individual subjects at 4 and 24 weeks: 10 subjects showed a substantial increase from week 4 to 24, while 7 subjects had little change and 3 demonstrated decreases in the proportion of virus precipitated at the 1:8 dilution (data not shown). At a 1:8 dilution of serum, all 20 subjects were positive by RIPA at week 4, while 19 (95%) of 20 were positive at week 24 (figure 4B, C). At a 1:800 dilution, 14 (70%) of 20 week 4 sera and 12 (60%) of 20 week 24 sera were positive. Following the booster immunization at week 24, sera from all 20 children were strongly positive by RIPA at all three dilutions tested (GMT, ≥1:800) (figure 4D). Preimmunization sera from most MRL-023 subjects were tested only at a 1:8 dilution in an effort to conserve the labeled antigen. At this dilution, none of the MRL-023 sera precipitated ≥30% of the virus (figure 4A).

**Correlation of RIPA titers with serum neutralizing antibody titers and antibody content of sera determined by modified HAVAB.** The titer of precipitating antibody determined by the semiquantitative RIPA (1:8, 1:80, and 1:800 dilutions) correlated well with other measures of anti-HAV response following immunization with inactivated HAV vaccine. Thirteen of 40 sera collected at weeks 4 or 24 after a single dose of vaccine in the MRL-023 study had relatively low but detectable RIPA activity (titer <1:80). Only 5 (38%) of these 13 sera tested positive by RIFIT, while 24 (92%) of 26 sera with high RIPA activity (titer ≥1:800) had RIFIT titers ≥1:8. Similarly, compared with sera that had low RIPA activity, sera with high RIPA activity had greater neutralizing antibody activity determined by HAVARNA (reciprocal GMT, 138 vs. 33.7) and greater antibody content determined by modified HAVAB.
Figure 3. Immunoprecipitation of radiolabeled HAV by antibodies present in sera collected prior to and 4 weeks after administration of single intramuscular dose of inactivated HAV vaccine. Left to right, bars for each study subject represent 1:8 and 1:80 dilutions of preimmunization serum specimen and 1:8 and 1:80 dilutions of paired postimmunization serum specimen. Study subjects were participants in MRL-020 trial.

(65.3 vs. 29.8 mIU/mL). High serum neutralizing antibody and HAVAB antibody titers at week 28, following booster immunization at 24 weeks, correlated with RIPA titers $\geq 1:800$ in each of the 20 subjects tested (table 1). In addition, booster immunization resulted in an increase in the fraction of labeled virus precipitated at each of the serum dilutions tested (cf. figure 4C and 4D).

Comparison of neutralizing antibody titers present following immunization with inactivated HAV vaccine and administration of IG. IG administered intramuscularly in a dose of 0.06 mL/kg results in highly effective but relatively short-term protection against clinical hepatitis A [5]. This protection is due exclusively to the presence of circulating, passively transferred antibodies to HAV. Thus, it was of interest to compare the levels of antibodies detectable after immunization with inactivated vaccine with those present after administration of IG. As shown in table 1, the level of antibody detectable by modified HAVAB shortly (7 days) after administration of IG to a group of 20 adults (MRL-005 study) was similar to that present in both groups of children studied 4 weeks after they were immunized with a single dose of inactivated vaccine: 45.1 mIU/mL vs. 49.3 (MRL-020, $P = .45$) and 45.2 mIU/mL (MRL-023, $P = .98$). However, all 20 of the IG recipients were positive for neutralizing antibodies by RIFIT, compared with only 34 (49%) of 70 vaccine recipients (table 1). Similarly, all 16 tested recipients of IG had neutralizing antibody detectable by HAVARNA. The reciprocal GMTs of neutralizing antibody measured by either assay were higher in IG recipients (27.5 and 146 for RIFIT and HAVARNA, respectively) than in vaccine recipients participating in the MRL-020 and MRL-023 studies (table 1). Thus, although immunoassay results indicated similar antibody levels in passively and actively immunized groups, significant differences were evident in the neutralization assays: $P < .01$ for RIFIT titers in either MRL-020 or MRL-023 (vaccine) versus MRL-005 (IG), while $P < .01$ and $P = .17$ for HAVARNA titers in MRL-020 and MRL-023 (vaccine) versus MRL-005 (IG), respectively. These results suggest that there may be qualitative differences in the antibodies present shortly after passive and active immunization, with lower levels of virus-neutralizing activity associated with similar antibody levels measured by modified HAVAB following immunization with inactivated HAV vaccine (cf. figures 5 and 2).

Antibody detected by RIPA following administration of IG. Sera collected from adult subjects in the MRL-005 study 7 days after administration of IG were tested for the presence of precipitating antibodies at 1.8 and 1.80 dilutions. Surprisingly, only 8 (40%) of 20 sera were positive, and none of these sera had RIPA titers $> 1.8$ (figure 6). The GMT of antibody detected by RIPA in sera collected after administration of IG was only 5.6, much lower than the GMT of $\geq 401$ found in sera collected 4 weeks after administration of inactivated HAV vaccine in the MRL-023 study ($P < .01$), even though these sera contained comparable titers of antibody measured by modified HAVAB ($P = .98$) (table 1). These results confirm that there are significant qualitative differences in circulating anti-HAV antibodies present following passive and active immunization that are not reflected in titers obtained with the modified HAVAB assay (table 1). These differences between subjects undergoing pas-
Figure 4. Percentage of radiolabeled virus precipitated in radioimmunoprecipitation assays (RIPA) using 3 different serum dilutions. Sera were from participants in Monroe, New York, efficacy trial (MRL-023) and were collected prior to (A) and 4 weeks after (B) priming dose of vaccine and prior to (C) and 4 weeks after (D) booster immunization 6 months later. Positive results were defined as ≥30% precipitation (dotted line).

Sive and active forms of immunization were particularly evident when the antibody titers of individual sera as determined by RIPA were compared with antibody content measured by modified HAVAB (figure 7).

The very low precipitating antibody activity found in IG recipients prompted us to consider whether the cold ethanol fractionation method used for the isolation of immunoglobulin from plasma pools during the manufacture of IG might be responsible for loss of activity in the RIPA. We therefore assayed two separate lots of IG (GAMMAR; Armour Pharmaceutical, Collegeville, PA) for precipitating antibody activity. Immunoglobulins present in both lots efficiently precipitated labeled virus at concentrations between 3 and 300 μg/mL (data not shown). However, these results are consistent with the low RIPA activity found in IG recipients. Assuming an even and complete distribution of passively transferred immunoglobulin within the extracellular fluid compartment (∼25% of body mass), expected peak serum concentrations of passively transferred IgG would be ∼40 μg/mL. Thus, at a 1:8 serum dilution, the maximum concentration of passively transferred IgG would be ∼5 μg/mL, which is at the lower end of the range of detection by RIPA. At a 1:80 serum dilution (∼0.5 μg/mL),
the quantity of transferred immunoglobulin would be below the limits of detection.

Discussion

The placebo-controlled trial of VAQTA carried out in Monroe, New York, by Werzberger et al. [2] (MRL-023) demonstrated a very high level of efficacy in prevention of clinical hepatitis A [2]. No cases of hepatitis A were observed among the immunized children in this study later than 16 days after intramuscular administration of a single 25-U vaccine dose, and statistically significant protection was evident by 30 days. To characterize the serologic correlates of protection induced by a single dose of this HAV vaccine, several different assays were used to measure antibodies to HAV in serum samples collected ~4 and 24 weeks after immunization from a randomly selected subset (n = 20) of the children who participated in this study. These 20 children were representative of the larger cohort of MRL-023 study participants as reflected by their age (mean, 7.8 vs. 7.9 years) and level of antibody measured by modified HAVAB assay 4 weeks after primary immunization (42 vs. 45.2 mIU/mL) (tables 1, 2) [2]. In addition, we also tested sera collected from a second cohort of children (MRL-020, n = 50) who had been immunized 4 weeks previously with the same dose of a different lot of this vaccine.

The GMT of antibody measured by solid-phase RIA (modified HAVAB) ranged from 45.2 to 52.9 mIU/mL in the various groups of sera tested (tables 1, 2), which is much lower than GMTs reported for seropositive persons who acquired antibodies to the virus following natural infection: 3485 mIU/mL [7] and 10,700 mIU/mL [19]. Similarly, titers of antibody measured by the neutralization assays were much lower than those present following natural infection [10, 11]. These low levels are near the limits of detection in the neutralization assays, leading to considerable interassay variability in titers obtained with individual sera. While this interassay variability detracts from the use of both the RIFIT and HAVARNA for measuring the early antibody response to inactivated HAV vaccine, these
assays provide unique information concerning the biologic activity of the anti-HAV antibody induced by immunization. Unfortunately, both of these assays are technically demanding and exceptionally labor-intensive.

The neutralization results summarized in tables 1 and 2 suggest that the RIFIT is considerably less sensitive than the HAVARNA. There are several reasons for this. First, RIFIT titers are based on the dilution of serum before mixing it with virus, while HAVARNA titers reflect the final dilution in the virus-serum mixture. These previously established conventions lead to a 2-fold inflation of HAVARNA titers. However, greater importance can be attributed to differences in the criteria for positivity in the assays: ≥80% reduction in radioimmunofoci in the RIFIT compared with ≥50% reduction in viral antigen expression in the HAVARNA. Fifty percent reduction end points can also be calculated for the RIFIT [10], resulting in considerably higher titers but reducing specificity at low serum dilutions. For example, the calculated GMT of neutralizing antibodies based on a 50% reduction of radioimmunofoci (50% RIFIT) with the 50 specimens collected from MRL-020 participants 4 weeks after immunization was 63.4 (data not shown), compared with 6.5 by the 80% RIFIT and 55.6 by HAVARNA (table 1). Only 2 of the 70 children studied in this report had 50% RIFIT titers ≤1:8 by 4 weeks after immunization. However, many HAVAB-negative preimmunization sera cause ≥50% nonspecific reduction of radioimmunofoci at a serum dilution of 1:8 (data not shown). In contrast, use of the 80% end point results in a very high level of specificity, as only 1 of 70 preimmunization sera generated a positive result by this criterion at a 1:8 dilution.

Nonspecific reductions in antigen expression are also observed in the HAVARNA. However, as used in this study, the HAVARNA was considered positive only if there was a 50% reduction in antigen expression compared with a parallel assay with a paired preimmunization sample. This allowed for high sensitivity with retention of good specificity but, unlike the RIFIT, required the testing of paired serum samples. In similar paired RIFITs, samples collected 4 weeks after immunization caused a ≥50% reduction in radioimmunofoci relative to preimmunization samples in only 41 (77%) of 53 children at a 1:8 serum dilution (data not shown). Since the HAVARNA was positive in 98% of children at this serum dilution (table 1), it is in fact more sensitive than the 50% RIFIT. This may be due to neutralization of second-cycle infections by residual antibody in the HAVARNA cultures, a phenomenon that would not affect the quantal RIFIT. Differences in RIFIT and HAVARNA titers are unlikely to reflect antigenic differences between the strains of HAV used in the two tests [20].

Previous reports have examined anti-HAV antibody levels present in serum following administration of IG and have suggested that similar antibody levels achieved following immunization with inactivated vaccines would be predictive of clinical efficacy [6, 7]. Indeed, the levels of antibody detected by modified HAVAB in serum from children who were protected by immunization with the inactivated HAV vaccine were similar to those present following administration of IG (45.1 mIU/mL, table 1). However, the neutralizing antibody levels as measured by RIFIT or HAVARNA were significantly lower in the immunized children than in the adult recipients of IG. Only 39 (56%) of 70 children were positive for neutralizing antibodies by RIFIT, while all 20 IG recipients tested positive by this assay (table 1). However, it is not surprising that the actively immunized children were protected despite their significantly lower neutralizing antibody responses. Neutralizing antibody activity

Figure 6. Percentage of precipitation of labeled virus obtained with sera collected 7 days after administration of immune globulin to adult volunteers (study MRL-005). Positive results were defined as ≥30% precipitation (dotted line).

Figure 7. Percentage of precipitation of radiolabeled virus in radioimmunoprecipitation assay (RIPA) plotted as function of antibody content determined by modified immunoassay (HAVAB) in serum samples collected 4 weeks after immunization of children in Monroe, New York, efficacy trial (○) (MRL-023) or 7 days after administration of immune globulin to adult volunteers (●) (MRL-005). Results are shown for 1:80 serum dilution only; mIU value is that of dilute specimen.
was measured in IG recipients 7 days after administration of the IG. Much lower activities would be expected several months later, at a time when residual levels of passively transferred antibodies still confer protection against disease [5, 21]. Only 16 of 19 sera collected from MRL-005 study participants 90 days after IG administration were reproducibly positive for neutralizing antibody by HAVARNA, and the GMT was 11.3 (data not shown).

The RIPA was by far the most sensitive method for detection of anti-HAV following immunization of children with inactivated HAV vaccine (table 1). However, while serum from 62 of 63 children obtained 4 weeks after a single dose of vaccine were positive in the RIPA, this was true for only 8 of 20 adult IG recipients (table 1). Moreover, while the GMT of precipitating antibodies in MRL-023 study subjects was $>1:401$ by 4 weeks after a single dose of the vaccine, none of the adult IG recipients tested positive by RIPA at a $>1:8$ serum dilution. Despite this, the GMTs determined by modified HAVAB titers were comparable following active or passive immunization, while the GMTs of neutralizing antibody in the IG recipients either equaled or exceeded those present in the immunized children (table 1). The contrasting RIPA activities, despite similar HAVAB and neutralizing antibody activities, suggest important differences in the affinity of the anti-HAV antibodies present after active and passive forms of immunization. Compared with the neutralization assays, which use very small virus inocula and are likely to be biased strongly toward detection of high-affinity antibody, the RIPA uses a very large amount of virus and is likely to be less sensitive to differences in antibody affinity under the conditions of the test. The sensitivity of the modified HAVAB assay (or similar competitive inhibition ELISAs) to differences in antibody affinity would be determined by the affinity of the labeled detector antibody. The lower affinity of vaccine-induced antibody suggested by these results is not unique to this particular HAV vaccine but has been noted also in recipients of the HM175 strain inactivated HAV vaccine (HAVRIX; SmithKline Beecham, Van Nuys, CA) [22].

We considered the possibility that the potent immunoprecipitating activity of serum samples from vaccine recipients might reflect the presence of HAV-specific IgM antibodies. However, a preliminary examination of the isotype specificities of immunoprecipitating antibodies indicated that RIPA activity in specimens collected 4 weeks after immunization was distributed across both 19S and 7S immunoglobulin classes, while the activity in 24-week samples was primarily associated with the 7S (IgG) fraction (data not shown).

Although technical considerations [23] precluded the preparation of sufficient quantities of the labeled virus for formal measurements of antibody affinity, these semiquantitative studies raise important practical considerations in the interpretation of antibody responses to HAV vaccines measured by solid-phase immunoassays in which the World Health Organization’s reference anti-HAV immunoglobulin is used as a reference [15]. This reference reagent contains convalescent antibody similar to that in IG, and thus its use as a standard must be viewed with caution when testing recent postimmunization sera for anti-HAV content. Similar quantitative levels of antibody activity measured in such assays may mask important biologic differences in antibody responses between patient groups, as we have demonstrated here. A reference reagent is needed that contains antibodies representative of those present following active immunization.

The qualitative differences between antibodies present in late convalescence (IG) and shortly after active immunization may reflect significant differences in antigen presentation, the total immunogenic mass of antigen, or simply the amount of time elapsed since exposure to antigen. They are unlikely to represent significant qualitative differences in the viral antigen itself. Although formalin inactivation has been shown to destroy some immunogenic epitopes on the surface of the closely related poliovirus [24], we have not identified consistent differences in the reactivities of multiple neutralizing monoclonal antibodies when these were tested in solid-phase immunoassays against native and formalin-inactivated HAV virions and empty capsids (Lemon SM, Lewis JA, unpublished data). It is likely that increases in antibody activity following booster immunization (table 2, figure 5) are associated with maturation of the antibody response and increased antibody affinity, although this was not assessed in this study. Such booster responses should enhance and extend the duration of the protective antibody response and are likely to be required for long-term protection following administration of inactivated HAV vaccine.

References


