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AM3 (Inmunoferón®) as an adjuvant to hepatitis B vaccination in hemodialysis patients

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Background. Patients with end-stage renal disease (ESRD) undergoing hemodialysis have severe alterations in cell-mediated immunity (CMI) that increases their risk of contracting chronic hepatitis B virus (HBV) infection and decreases their protective responses to HBV vaccine. In an effort to improve the humoral response to an accelerated HBV vaccine protocol in these patients, the ability of an immunomodulator, AM3, to improve seroconversion was investigated.

Methods. A total of 269 patients were enrolled in a multicenter trial. All patients received a DNA recombinant vaccine (40 μg HBsAg/dose/day) on days 0, 10, 21, and 90. AM3 or placebo (3 g/day) was given orally for 30 consecutive days beginning 15 days prior to the first vaccine dose. Anti-HBsAg titers were measured on days 120 and 270 after the beginning of the trial.

Results. After one month, 207 patients could be evaluated and 132 patients after six months. The placebo and AM3-treated groups had comparable seroconversion and protective response rates one month after the final vaccine dose. The AM3 treatment group, but not the placebo group, maintained these protective titers up to six months after the final vaccine dose. At this time, the percentage of high responders (anti-HBsAg >100 IU/L) and mean anti-HBsAg titers in the AM3 group was significantly higher than in the placebo group.

Conclusions. AM3 is a safe and easily tolerated oral agent that potentiates long-term serological immunity to hepatitis B in hemodialysis patients after vaccination.

In end-stage renal disease (ESRD) patients treated with hemodialysis, the immune response is impaired and an associated state of chronic inflammation exists [1]. High susceptibility to infections accounts for 36% of the mortality of ESRD [2]. Despite the use of universal precaution techniques in hemodialysis centers, hepatitis B vaccination, and the reduction in the need of transfusions, ESRD patients are still at increased risk of hepatitis B virus (HBV) infection. This contributes significantly to morbidity and mortality [3].

Unfortunately, 30 to 40% of hemodialyzed ESRD patients neither seroconvert nor develop protective anti-HBV responses to hepatitis B vaccination [3–10]. Further, the duration of any immunity developed is shorter [11, 12], and the secondary immune responses to revaccination clearly lower than those observed in healthy people [13–15]. This suggests that immunological memory is not firmly established at the time of the first vaccination. The failure to seroconvert is influenced by age and the degree of renal failure [16]. It also has been associated with a number of immunological parameters such as dysfunctional T-cell responses and decreased in vitro T-cell proliferation in response to hepatitis B surface antigen (HbsAg) [17], altered cytokine production [18–21], and decreased expressions of TCR/CD3, the interleukin-2 (IL-2) receptor, and human lymphocyte antigen-DR (HLA-DR) [17].

Attempts to increase HBV seroconversion in vaccinated, hemodialyzed patients with adjuvant signals such as cytokine IL-2 [22–25], granulocyte-macrophage colony-stimulating factor (GM-CSF) [26, 27] and interferon gamma (IFN-γ) [28] have had no clear effect. Previous efforts to improve the response to HBV vaccination by stimulating T-cell and antigen-presenting cell function with immunomodulators (such as thymopentin) have met with varying degrees of success. The efficacy of thymopentin as an adjuvant to HBV vaccine is controversial. Some studies show it to provide either no [29–31] or only modest [32] benefit.

The immunomodulator AM3 (Inmunoferón®) is a clinically used, orally administered polysaccharide/protein compound purified from *Candida utilis* that has regu-

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latory effects on the production of pro-inflammatory cytokines [33–37]. It also increases the absolute number and activity of natural killer (NK) cells [33–35] and increases both phagocytic cell activity and anti-tumor activity [35]. In animal models, the capacity of AM3 to potentiate natural (innate) and specific immunity is related to the induction of endogenous production of IL-12 and IFN-γ [36, 37] while partially inhibiting the production of tumor necrosis factor alpha (TNF-α) [37]. AM3's ability to clinically improve immune activity has been confirmed by reductions in respiratory infections in adult patients with inflammatory bronchial disease [38] as well as by the restoration of cutaneous delayed-type hypersensitivity reactions in children with asthma [39], reductions in recurrent aphthous ulcers in oral stomatitis [40], and in improved viral clearance in chronic hepatitis B carriers [41, 42]. Further, AM3 has been shown to be an effective adjuvant in hepatitis B vaccination in healthy people who previously failed to develop HBsAg titers $>$10 IU/mL in response to the recombinant hepatitis B vaccine [36, 43] as well as in hemodialysis patients who were non-responders to hepatitis B vaccination. The exact mechanism of the immunostimulation effect of AM3 in these different settings is not well known, although some studies suggest that a complex process of induction of immunoregulatory cytokines is involved [36, 37].

The ability of AM3 to act as an immunoadjuvant in healthy non-responders to hepatitis B vaccination suggests that it may be helpful in hemodialyzed ESRD patients as well. AM3's stimulatory effects on cell-mediated and innate immune responses may allow ESRD patients to develop more effective antibody responses to HBV vaccination. The adjuvant effect of AM3 on the response to hepatitis B was therefore evaluated in a randomized, placebo-controlled, double-blind clinical trial of hemodialyzed ESRD patients.

METHODS

Protocol

Subjects. Prior to their inclusion, all recruited subjects received concise information about the study, were given the opportunity to ask questions, and gave their witnessed, informed consent in writing. The study was approved by all the ethical committees of the hospitals involved as well as by their respective national health authorities. Initial admission studies for inclusion of each patient involved a brief medical history, a physical examination, a complete hemogram, serological studies of hepatitis B surface and core antigens and antibodies, serum electrolyte studies, evaluation of glucose levels, liver function tests, and the determination of calcium, magnesium, cholesterol, triglyceride, albumin and uric acid levels. When feasible, serology and hepatitis C RNA detection also were undertaken.

Inclusion criteria. Individuals of both sexes between the ages of 18 and 80 years with a diagnosis of ESRD, who received hemodialysis but who had no detectable anti-HBsAg titers were eligible for inclusion. These subjects had either never received hepatitis B vaccination or were repeatedly unable to achieve detectable anti-HBs titers after hepatitis B vaccination. Inclusion criteria also demanded that subjects have undetectable anti-HBs titers at entry point, and have no serological evidence of hepatitis B infection [absence of positive serology for hepatitis surface and core antigens (HBsAg, HBeAg), antibodies to core antigens (anti-HBc), or elevated levels of hepatic transaminases (AST, ALT)]. Further, subjects had to be able to understand and comply with the study protocol and to sign a note of informed consent.

Exclusion criteria. The following subjects were excluded from the study: those unable or unlikely to comply with the study protocol (for example, drug or alcohol dependent), pregnant or breast feeding mothers, women who rejected the use of standard birth control methods, those with previously identified allergies to the hepatitis B vaccine, Inmunoferón® (AM3) or one of its components, subjects with serology positive for hepatitis B (HBsAg, HBeAg, anti-HBc), those who showed a humoral response to hepatitis B vaccination greater than 0 IU/L, those with a congenital or acquired immunodeficiency or autoimmune disease, those who in the three months prior to the study had received immunosuppressants, immunomodulators, cimetidine or other medications capable of modifying the immune response, and, finally, those who showed evidence of intestinal malabsorption.

Interventions. The study design is outlined in Figure 1. Hepatitis B vaccine (Recombivax HB, Pasteur Merieux, MSD Laboratories, Lyon, France) containing 40 μg of HBsAg was administered IM (deltoid) following an accelerated schedule (0, 10, 21, and 90 days). All doses were from the same lot to diminish variation in antigenicity. Subjects were kept under medical supervision for hypersensitivity reactions for half an hour after each administration. Subjects began to take AM3 or placebo 15 days (d $-$ 14) prior to receiving the first 40 μg dose of hepatitis B vaccine (d 0). AM3 (Inmunoferón®; I.F. Cantabria, Madrid, Spain) is an oral immunomodulator with a low toxicity profile. Previous dose-finding and kinetic studies with AM3 in pilot studies demonstrated that 3 g/day is the optimal dose for maximal immunostimulation without side effects. AM3 or placebo was given by the oral route at doses of six capsules per day (2 capsules three times a day) for 30 consecutive days. Whole blood was drawn into vacutainer tubes for serum collection at baseline (d 0), 30 days after patients received the final dose of hepatitis B vaccine (d + 120), and six months after the final vaccine dose (d + 270). Anti-HBsAg antibody titers, expressed as IU/L, were analyzed by enzyme-linked immunoassay (ELISA; Ausab, AxSYM; Abbot

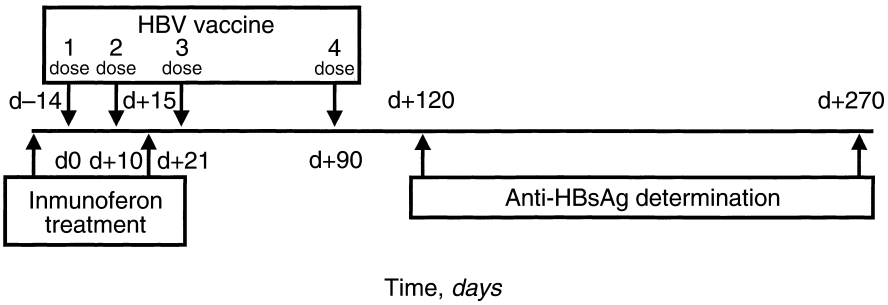

Fig. 1. Study outline.

Laboratories, Berkley, CA, USA) with a sensitivity of <1 IU/L.

Outcome measures. The ability of AM3 to potentiate the antibody response to hepatitis B vaccine was measured at one month and six months after the end of the accelerated vaccination protocol. Efficacy was determined by the percentage of the vaccinated population in either group achieving seroconversion (>10 IU/L) or who had protective (>100 IU/L) anti-HBsAg titers, as well as the concentration of anti-HBsAg titers among subjects with detectable levels. Our unpublished data from previous pilot studies indicate that, in response to hepatitis B vaccination, AM3 treatment increases seroconversion from 30% in the placebo group to 60% in the AM3 treated group in healthy people who did not respond to previous vaccination. Thus, for this study, sample sizes of at least 58 patients per group were needed to detect a difference of 30% with a standard deviation of 10% at 95% power with alpha set at 0.05. It was estimated that 10% of patients would be lost owing to follow-up or protocol violations. Subjects who had consumed at least 80% of the capsules (placebo or AM3) were considered in the final evaluation of AM3 efficacy.

Participant flow and follow-up

Subjects were recruited from 17 European centers. Two hundred and sixty-nine patients were included in the study and randomized to either the placebo (N = 132) or AM3 (N = 137) group (Fig. 2). Of the 269 patients with safety data, 62 withdrew prior to evaluation of efficacy at one month following the final vaccine dose (28 placebo, 34 AM3). An additional 75 subjects withdrew prior to follow-up six months after the final vaccine dose (37 placebo, 38 AM3). Reasons for withdrawal were equally represented in the AM3 and placebo treatment groups (Table 1). Only four patients from each treatment group withdrew for adverse effects. Forty-two patients were excluded from efficacy analysis because of protocol violations (7 for age >80; 11 for anti-HBsAg titers >0 IU/L; 13 for steroid or other immunosuppressive treatments in the 3 months prior to the study or during the follow-up; and 11 for non-compliance). The baseline characteristics of the intention-to-treat population are shown in Tables 2 and 3. The treatment groups were similar across all the measured variables with the exception that the AM3 group contained a significantly larger number of males (and thus showed a mean height significantly higher than the placebo group), and that the placebo group had a significantly greater number of subjects receiving erythropoietin treatment. The 103 patients who were included in the population used for evaluation of efficacy had similar distributions among baseline characteristics. The significantly higher proportions of males in the AM3 group (P = 0.01), and of erythropoietin treated patients in the placebo group (P = 0.02), were maintained. The treatment groups did not differ with respect to nutritional status (weight-for-frame size and serum albumin; Table 2), and there were no significant differences in the dialysis adequacy (Kt/V) between groups. Importantly, the groups did not differ with respect to mean time spent in dialysis in either the intention-to-treat population or that used for the evaluation of efficacy.

Analysis

Safety of AM3 treatment. Adverse events were monitored at each of the vaccine dose administrations (days 0, +10, +21, +90) as well as over the course of the follow-up. Subjects were instructed to note any adverse events when they recorded their daily intake of treatment capsules. Twenty-five (18.2%) of the AM3 group and 20 (15.1%) of the placebo group reported adverse effects (Table 4). Mild gastrointestinal symptoms were the only adverse effects likely to be related to the administration of AM3. Biochemical and hematological parameters measured at baseline, immediately after completing AM3 or placebo treatment, and at the one month follow-up, were not significantly changed either in the AM3 or placebo group (data not shown).

Statistical analysis. All analyses were performed using SAS for Windows (SAS Inc., Cary, NC, USA). In order to ensure accuracy, subject data were entered by two different people into two different databases. The resulting databases were compared and merged to form a final copy. Normality was determined by the Shapiro Wilks test for sample sizes below 200. Differences between placebo

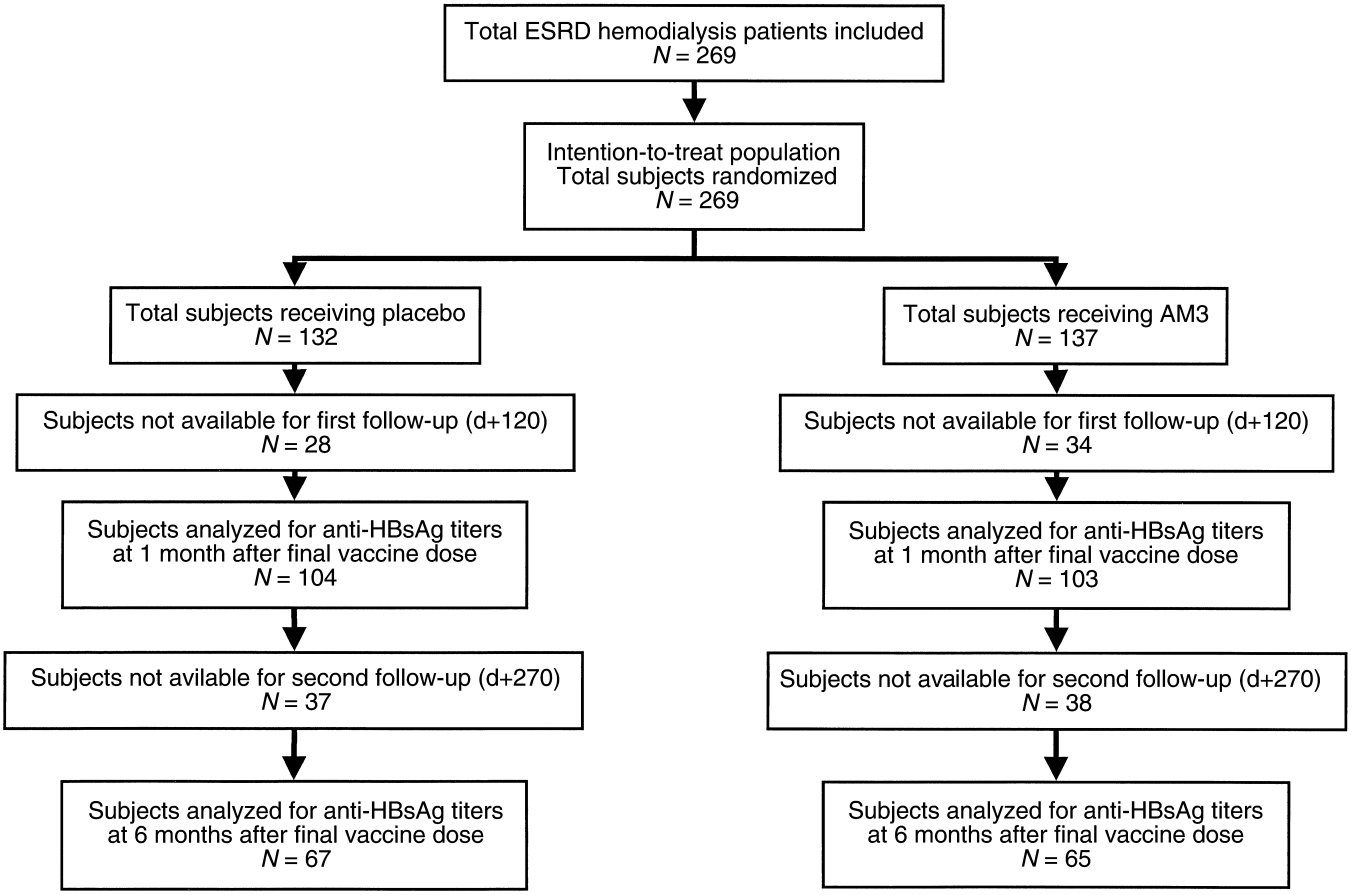

Fig. 2. Trial profile.

Table 1. Reasons for withdrawal from study at each end point

	One month follow-up		Six month follow-up	
	Placebo	AM3	Placebo	AM3
Protocol violations				
Age >80 years	2 (1.5%)	5 (3.6%)	0	0
Previous anti-HBsAg titers >0 IU/L after primary vaccination or previous hepatitis B vaccination or anti-HBsAg titers >0 IU/L at entrance into study	6 (4.5%)	5 (3.6%)	0	0
Treatment with steroids or other immunosuppressors within three months of inclusion in the study or during the follow-up	0	1 (0.7%)	5 (3.7%)	7 (5.1%)
Non-compliance with treatment protocol	7 (5.3%)	4 (2.9%)	0	0
Voluntary withdrawal	3 (2.2%)	5 (3.6%)	15 (11.3%)	13 (9.4%)
Death	3 (2.2%)	4 (2.9%)	5 (3.7%)	7 (5.1%)
Renal transplant	3 (2.2%)	6 (4.3%)	2 (1.5%)	1 (0.7%)
Adverse effects	4 (3.0%)	4 (2.9%)	0	0
Transferred to another center	0	0	10 (7.5%)	10 (7.2%)
Total	28 (20.9%)	34 (24.5%)	37 (28.0%)	38 (27.7%)

and treatment groups were compared by the two-tailed Student *t* test or, when appropriate, by the nonparametric Mann Whitney U test. Comparisons of categorical variables were performed with the aid of the Chi squared test or, when appropriate, Fisher's Exact test. Significance was set at $P \leq 0.05$ for all analyses. To evaluate the impact of patients lost to follow-up or protocol violations, comparisons were completed on an intention-to-treat basis.

Assignment

Randomization was performed in blocks of four using a program created using Clipper v. 5.2. One of the two

Table 2. Demographic and baseline information of the intention-to-treat population

Subject characteristics	Placebo	AM3	*P*
Gender			0.004
Male	67 (50.8%)	93 (67.9%)	
Female	65 (49.2%)	44 (32.1%)	
Age in years	132 (60.4 ± 14.7)	137 (60.5 ± 15.0)	0.947
Race			—
Caucasian	128 (96.9%)	133 (97.1%)	
Other	4 (3.1%)	4 (2.9%)	
Height *cm*	125 (162.5 ± 8.3)	129 (165 ± 8.5)	0.019
Weight *kg*	131 (64.7 ± 12.1)	134 (66.7 ± 12.9)	0.204
Receiving erythropoietin treatment	113 (85.6%)	103 (75.2%)	0.032
Previously vaccinated against hepatitis B	25 (18.4%)	34 (24.8%)	0.244
Positive for antibodies for hepatitis C (measured in 260)	15 (11.7%)	19 (14.4%)	0.522
Positive for hepatitis C RNA (measured in 64)	8 (26.7%)	9 (26.5%)	1.0
Albumin *g/dL*	4.0 ± 0.6	3.9 ± 0.4	0.782
Months on hemodialysis	122 (38.4 ± 41.0)	133 (35.3 ± 38.9)	0.540

Data are shown as the *N* (%) or *N* (mean ± SD) as appropriate.

Table 3. Etiology of renal failure in the intention-to-treat population

	Placebo	AM3	Total
ESRD etiology		*N* (%)	
Primary renal	52 (39.4%)	53 (38.7%)	105 (39.0%)
Secondary renal			
Diabetes	20 (15.1%)	29 (21.2%)	49 (18.2%)
Vascular/hypertensive disease	22 (16.7%)	25 (18.2%)	47 (17.5%)
Other systemic disease	5 (3.8%)	3 (2.2%)	8 (3.0%)
Interstitial nephritis secondary to analgesics	3 (2.3%)	4 (2.9%)	7 (2.6%)
Chronic renal insufficiency of unknown etiology	30 (22.7%)	23 (16.8%)	53 (19.7%)
Total	132	137	269

Table 4. Adverse events in the intention-to-treat population

Adverse events	Placebo *N* = 132	AM3 *N* = 137
Mild gastrointestinal symptoms	6 (4.5%)	4 (2.9%)
Local anaphylactic reaction to vaccination	2 (1.5%)	0
Technical complications of hemodialysis	1 (0.7%)	2 (1.4%)
Arteriovenous graft infection/thrombosis	3 (2.2%)	5 (3.6%)
Anemia	0	1 (0.7%)
Cerebral ischemic accident	0	1 (0.7%)
Death	8 (6.0%)	11 (8.0%)
Vertebral fracture and aspiration pneumonia	0	1 (0.7%)
Total	20 (15.1%)	25 (18.2%)

possible treatments (placebo or AM3) was assigned to a list of random numbers. The drug was labeled with a number and distributed to each hemodialysis or medical center in batches of consecutively numbered sets of two boxes of medicine each. Upon inclusion, each subject was assigned medication in his/her hemodialysis or medical center corresponding to the lowest number available. For example, if the center had sets numbered 001 through 016, the first patient would receive 001, the second would receive 002, etc. There were no code violations during the study period. The code was broken after all subject data were collected, just prior to sending them for off-site statistical analysis.

Masking

Each patient was given two boxes with 180 capsules in individual blister packs as well as a medication diary to record each dose taken. The capsules contained either 500 mg of AM3 or placebo (both are flavorless, white powders). The boxes, the labels on the boxes, and the capsules for placebo or AM3 were indistinguishable to both investigator and subject. At the end of the 30-day treatment period, subjects handed in their medication diaries and boxes with blister packs. Investigators and subjects were therefore blinded to the protocol throughout the study period. In an effort to reduce bias, statistical analysis was contracted out to an unrelated party not blind to the treatment group.

RESULTS

Serological response to hepatitis B vaccination

The adjuvant effect of AM3 on the serological response to the accelerated hepatitis B vaccine protocol was determined by comparing anti-HBsAg titers at one and six months following the final vaccine dose adminis-

Table 5. Serological response to an accelerated protocol of hepatitis B vaccine

	One month follow-up		Six month follow-up	
	Placebo $N = 104$	AM3 $N = 103$	Placebo $N = 67$	AM3 $N = 65$
Seroconversion (anti-HBsAg >10 IU/L)	65 (62.5%)	72 (69.9%)	42 (62.7%)	42 (64.6%)
Protective response (anti-HBsAg >100 IU/L)	44 (42.3%)	53 (51.5%)	18 (26.9%)	30 (46.2%)[a]
Low response (10 < anti-HBsAg <100 IU/L)	21 (20.2%)	19 (18.4%)	24 (35.8%)	12 (18.4%)
Mean ± SD anti-HBsAg titer of those with titers >0 IU/L	624.9 ± 1149.6	675.7 ± 1389.0	199.7 ± 285.9	330.4 ± 340.2[b]

[a] Significantly more subjects from AM3 group than placebo group developed protective responses, $P = 0.03$
[b] Mean anti-HBsAg titers are significantly greater in AM3 group as compared to placebo, $P < 0.02$

tered (Table 5). Both treatment groups had rates of seroconversion greater than 60% at one and six months after the final vaccine dose. No significant differences were seen in the rates of seroconversion between treatment groups. Further, at the one month follow-up, 53 out of 103 patients on AM3, and 44 out of 104 patients on placebo, developed protective titers (anti-HBsAg >100 IU/L). While seroconversion rates were relatively well maintained up to six months after the final vaccine dose, the percentage of subjects with protective titers fell significantly in the placebo group. While the AM3 group had 46.2% (30 of 65) subjects with protective titers, the placebo group at the final follow-up included only 26.9% (18 of 67) subjects with protective titers ($P = 0.03$). In addition, while both groups demonstrated declines in mean titers from the one month to the six month follow-up, the AM3 group had significantly higher mean titers than did the placebo group at six months after the final vaccine dose. At one and six months after the final vaccine dose, there were no significant differences between groups of patients with respect to low titers of anti-HBsAg antibodies (10 < anti-HBsAg <100 IU/L). In the group of 100 naïve vaccinated patients, those treated with AM3 showed a significantly better response to the hepatitis B vaccination (26 developed protective response, 7 low titers and 13 did not seroconvert) than those treated with the placebo (15 developed protective response, 21 low titers and 18 did not seroconvert; $P = 0.006$). There were no significant differences between the AM3 and placebo treatments with respect to the rates and levels of seroconversion in the 32 patients who had failed in former vaccination protocols.

The number of patients lost to follow-up in the population used for evaluation of efficacy was similar in the two patient groups (Fig. 2). Comparative analysis of the rates of seroconversion, protective titers and mean anti-HBsAg titers of those patients who did not reach the six-month endpoint (N = 75) showed no significant differences between the AM3 and placebo groups at one month following vaccination (data not shown). Further, we analyzed the rates of seroconversion, protective titers and mean anti-HBsAg titers of the patients lost after the first month of follow-up and those remaining in the study. There were no significant differences in these vaccination response parameters between such patients in both treated groups after the first month of study (data not shown). These findings, combined with the parallel loss of subjects in both treatment groups, argue against any bias being introduced by subject withdrawal.

DISCUSSION

This randomized, placebo-controlled clinical trial demonstrates a clear role for AM3 as an adjuvant to hepatitis B vaccination in hemodialyzed ESRD patients for the development of prolonged anti-HBsAg protective titers. The clinical relevance of achieving anti-HBsAg titers >100 IU/L after vaccination has been well established in immunocompromised patients [7, 12, 44, 45] as well as in infants at high risk of hepatitis B infection [46]. Further, some authors [51], as well as some countries (United Kingdom) [45–48], have recommended adopting 100 IU/L as the minimum protective titer against a hazardous hepatitis B infection in healthy people.

Hemodialyzed ESRD patients achieve lower antibody titers after vaccination, lose antibody titers more rapidly, and have a higher risk of natural infection after vaccination than do healthy subjects [4, 8, 12]. Since increased cell-mediated immune (CMI) responses against hepatitis B are associated with higher humoral responses to vaccination [49–51], it is reasonable that an adjuvant that supports CMI function such as AM3 [35–37] would improve long-term serological immunity following vaccination.

Two factors varied unequally between the placebo and AM3 groups. The latter showed a significantly greater percentage of males than did the placebo group. Previous studies have demonstrated that hemodialyzed females [9], as well as healthy adult women [49, 52], have significantly better serological responses to vaccination than do men. The greater percentage of men in the AM3 group (Table 2) could therefore have introduced a bias against the efficacy of AM3. Secondly, although the use of erythropoietin was high in both treatment groups, the placebo group had a significantly greater number of subjects receiving this medication than did the AM3 group (Table 2).

Whether or not erythropoietin has an effect on seroconversion rates after hepatitis B vaccination has not been not established since controlled studies are lacking [7, 44, 53]. Most studies indicate a stimulatory effect on serological responses to hepatitis B vaccine. Again, the greater representation of erythropoietin treatment in the placebo group could have introduced a possible bias against AM3's efficacy. Finally, it has been suggested that patients with positive hepatitis C serology, but not with positive RNA, have a lower hepatitis B antibody response after vaccination [45, 54, 55]. Comparisons of hepatitis C antibodies and RNA demonstrated similar levels between the treatment groups. It is therefore to be expected that any inhibitory effect would occur equally in the AM3 and placebo groups.

To our knowledge, this is the first report of the administration of an immunomodulator resulting in a clear and prolonged antibody response flowing hepatitis B vaccination in ESRD hemodialyzed patients. Cytokines such as IL-2 [23] or GM-CSF [56, 57], and immunomodulators such as thymopentin [29, 30], have failed as adjuvants in this patient population. The benefit of AM3 as a safe, short-term oral adjuvant, with few or no side effects in this immunocompromised population, is promising. The significant increase in the percentage of patients with protective titers of anti-HBsAg antibodies induced by the adjuvant use of AM3 is clinically important in this high-risk patient population. The efficient effect of AM3 in increasing the percentage of naïve vaccinated patients who conserved a protective response after six months of follow-up, support the use of this immunomodulator as an adjuvant with hepatitis B vaccination in this population of ESRD patients. While it is very likely that the preservation of antibody titers seen in this study is due to the establishment of long-term immunological memory owing to the influence of improved CMI function, further studies are necessary to address this fully.

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