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MODULATION OF IMMUNE RESPONSE TO rDNA HEPATITIS B VACCINATION BY PSYCHOLOGICAL STRESS

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Abstract—In a previous study it was shown that antibody formation after vaccination with a low-dose recombinant DNA (rDNA) hepatitis B vaccine was negatively influenced by psychological stress. The present study was designed to assess whether the same inverse relation between HBs-antibody levels and psychological stress could be observed, while administering the standard, and thus higher, dose of vaccine. Volunteers ($n = 68$) scoring extremely low or high on a combination of questionnaires measuring daily problems and psychoneurotic symptoms were selected for participation. Antibody levels were determined 2, 6, and 7 months after the first vaccination. Questionnaires were completed before entering the study and at month 6. In contrast to the previous study, psychological stress was not found to be related to the antibody levels at any timepoint. These results suggest that, under certain conditions, stress-induced immunomodulation *in vivo* might be dependent on antigen dose.

Keywords: Vaccination; Hepatitis B; Psychological stress; Psychoneuroimmunology

INTRODUCTION

Nowadays it is well established that psychological factors can influence immune functioning. Until now research in psychoneuroimmunology (PNI) has largely focused on *in vitro* immune parameters. However, such *in vitro* parameters do not always have unequivocal relevance in terms of clinical practice. *In vivo* immune reactivity, (e.g., after an immunization or antigenic challenge) can yield more relevant information in this respect. Cohen and Williamson [1] have emphasized the need for prospective infectious-challenge studies to evaluate the role of psychological stress in disease susceptibility and course of clinical manifestations. Prospective studies allow the experimenter to control for variables like previous exposure, antigen dose, infection rate, disease onset, and symptomatology. With a few exceptions, such studies have

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not been performed in the field of PNI. Studies performed thus far, have focused mainly on infections of the upper respiratory tract and herpesvirus infections. These studies have shown that introversion [2, 3], psychosocial load [2–5], and emotional dysfunction [5–8] are related to the manifestation of these infections.

Ethical constraints, particularly in humans, limit the possibility of studying experimentally induced infections. This constraint can be circumvented by using nonpathogenic antigenic stimuli, such as those applied in vaccinations. An indication of the functioning of the integrated system is given by antibody levels thus evoked or cellular responses as measured by the proliferative capacity of the lymphocytes. Only a few human studies have been performed using this paradigm. After the vaccination of students with trivalent influenza vaccine, Bovbjerg *et al.* [9] demonstrated a negative relationship between distress and the level of proliferative response of blood lymphocytes to the influenza virus *in vitro*. Locke *et al.* [10] used a trivalent influenza vaccine as well, but failed to find any associations between antibody levels after immunization and psychological variables such as life stress, depression, anxiety, and helplessness.

Antibody formation after hepatitis B vaccination has been measured in some previous studies on stress-induced immunomodulation. Contrary to expectations, Petry *et al.* [11] reported a positive correlation between peak antibody titers and negatively perceived stressful events, irascibility, depression, and anxiety during the induction phase of immunization. Glaser *et al.* [12] vaccinated students on the first of a 3-day examination period. Seroconversion after the first vaccination was found to be negatively related to anxiety and self-reported stressfulness of life situations. We have previously reported that hepatitis B vaccination in subjects participating in a study on the immunogenicity of low-dose rDNA hepatitis B vaccine has yielded corresponding data [13]. Chronic psychological stress reported early in the study (month 2) was inversely related to antibody levels 7 months after immunization. Although associations were in the same direction for stress scores measured at month 6, these failed to reach statistical significance. These findings led us to hypothesize that psychological variables influence antibody formation mainly during the induction phase of the immune response, when memory cells are generated. This may explain why the booster vaccination 6 months after priming results in lower antibody levels.

Our previous study contained two rather unique elements. First, we applied measures of self-reported chronic psychological stress as evaluated from questionnaires, instead of groups of subjects exposed to specific stressful life episodes such as academic exams, divorce, or the death of a loved one. Second, the dose of the vaccine administered to the volunteers was considerably lower than those usually applied in regular vaccination programs. In rodents, suboptimal dosing of antigen is recommended for the detection of stress effects on immune reactivity [14]. The present study was undertaken to determine whether the previously observed inverse relation between HBs-antibody levels and reported psychological stress is maintained, now applying the vaccine dose administered regularly in vaccination programs. To optimize the experimental conditions, the study was performed using a group of students who scored extremely low or high on a combination of stress questionnaires. It was hypothesized that, as in the previous study, HBs antibody levels of highly stressed individuals are lower than those of subjects scoring low on the stress questionnaires, especially during the induction phase of immunization.

Table I. – Group means for the EPCL and HSCL at selection (month 0)

	EPCL*	HSCL*	Number of subjects
Low/low	16.0 (±4.9)	64.2 (2.7)	19
Low/high	27.1 (9.4)	91.4 (13.5)	18
High/low	78.3 (27.3)	76.7 (6.4)	15
High/high	97.9 (38.0)	113.4 (15.9)	16

Groups: low/low: low score on EPCL and low score on HSCL; low/high: low score on EPCL and high score on HSCL; high/low: high score on EPCL and low score on HSCL; high/high: high score on EPCL and high score on HSCL.

*Values expressed as mean(s), standard deviation(s) in parentheses.

METHOD

Subjects

Sixty-eight students (43 women and 25 men with a mean age of 21.8 ± 3.0 years) volunteered for the vaccination study. They were selected from a population of 390 Utrecht University students of different curricula in their second and third year, all of whom had completed the selection questionnaires. The questionnaires included measures of daily problems, symptoms, and biographical data. To maximize the chance of finding effects of psychological variables, subjects scoring extremely low or high on the psychological questionnaires were asked to participate in the vaccination study. Potential participants had to meet one of the following four criteria: a low or high score on both selection tools (Everyday Problem Checklist [EPCL] [15] and the Hopkins Symptom Checklist [HSCL] [16]); a low score on the EPCL but a high score on the HSCL; or a high score on the EPCL combined with a low score on the HSCL.

Because the EPCL and the HSCL are correlated ($r = 0.59$–0.62) selection criteria for the low/low groups and high/high groups were more extreme (10% lowest and highest) than for the low/high and high/low groups (30% lowest and highest). Group means for the EPCL and HSCL at selection (month 0) are shown in Table I. Subjects were informed on the vaccination and blood drawing procedures and asked whether they were prepared to complete the questionnaires once more. Before entering the study, informed consent was obtained from all participants.

Subjects were checked for abnormalities in health status or medicinal drug use. This led to the removal of one subject suffering from psoriasis. There were equal numbers of males and females among the four groups.

Psychological measures

The following questionnaires were used in the study:

- The Everyday Problem Checklist (EPCL) [15, 17], measured the frequency and intensity of chronic and everyday stressors during the 2 months prior to questionnaire completion. The EPCL is a Dutch 114-item checklist with proven validity and reliability containing items from the following domains: (1) family life; (2) living conditions; (3) working conditions; (4) physical appearance and own performance; (5) transactions and business; (6) social life; and (7) confrontations.
- The Hopkins Symptom Checklist (HSCL) [16, 18], assessed psychoneurotic and psychosomatic symptoms during the week preceding questionnaire completion;
- A questionnaire assessed health and biographical data including gender, age, alcohol use, smoking habits, body weight, and height.

Questionnaires were filled out twice: as a selection instrument before entering the study and 6 months after the first vaccination. They were encoded, completed anonymously, and returned by mail.

Factor analyses on the EPCL and HSCL resulted in one principal component, suggesting one underlying concept. Following Cohen *et al.* [19], two stress index scores combining the two questionnaires were calculated, representing the degree of psychological stress experienced by the subjects at the beginning and at the end of the study:

1. A stress index score-month-0 (SI-0): Using the EPCL and HSCL sum scores of month 0, a single

principal component was obtained accounting for 79% of the variance. Both questionnaires loaded 0.89 on this factor.

2. A stress index score-month-6 (SI-6): Using the EPCL and HSCL completed at month 6, a single principal component was obtained accounting for 81% of the variance. The questionnaires loaded 0.90 on this factor.

These stress index scores (i.e., factor scores based on principal components analyses) were used in the statistical analyses.

Using body weight and height measures, Quetelet indices were calculated for each participant, indicating over- or underweight status.

Vaccination

A recombinant DNA hepatitis B vaccine (HB-VAX-DNA; Merck Sharp & Dohme) was administered intramuscularly in the triceps muscle. The standard immunization protocol was applied, including vaccinations of a dose of 10 micrograms at months 0, 1, and 6. All vaccines used in the study were from one single batch.

Antibody levels

Antibody levels to hepatitis B surface antigen (anti-HBs) were determined with a direct noncompetitive "sandwich" assay based on an enzyme-linked immunosorbent assay (ABAU-STD-SET, Sorin Biomedica, Saluggia, Italy). Plasma samples were incubated overnight at room temperature in wells coated with HBsAg. After washing the wells, an enzyme tracer, consisting of HBsAg conjugated to horseradish peroxidase, was added and incubated for 4 hours at room temperature. Enzyme activity was measured by adding a chromogen/substrate dilution of tetramethylbenzidine, 0.005% H_2O_2, and citrate buffer. Results were read with a spectrophotometer at 450 nm after adding sulfuric acid as a blocking reagent.

Antibodies were determined four times during the vaccination program: at months 0, 2, 6, and 7, and quantified using the WHO standard preparation [20]. All assays were read in a blind coded manner. At time 0, anti-HBs levels from all participants were negative. Only subjects with a detectable antibody level ($\geq$1 IU/l) after vaccination were included in statistical analyses.

Statistical analyses

Because anti-HBs titers were not distributed normally, data were log-transformed. Although an extreme group design was used, the changes in scores on EPCL and HSCL over time prevented us from applying analysis of variance. Instead, hierarchical multiple regression analyses were performed to assess which variables influence antibody levels. Groups of variables were entered stepwise in the equation. Once (groups of) variables were entered, they remained in the regression equation. To reduce variance and enhance the sensitivity of the design, for the analyses on antibody concentrations of months 6 and 7, the antibody levels of the preceding assessment (anti-HBs-2 and anti-HBs-6, respectively) were entered in step 1. Lifestyle variables, such as smoking and alcohol consumption are known to influence antibody concentrations following vaccination [21]. Body mass is expected to influence uptake of the vaccine and therefore the resulting antibody level [21]. Gender is known to affect antibody formation *in vivo*, with women having higher antibody levels than men [21]. To control for these variables, the number of cigarettes smoked daily, weekly alcohol consumption, Quetelet index, and gender (0 = male, 1 = female) were entered in step 2. In the last step, the psychological stress measures (SI-0 and SI-6) were entered independently. In step 3a, SI-0 was entered. In step 3b, SI-6 was entered instead of SI-0. With the *F*-test for model comparison it was calculated whether the stepwise addition of new variables in the equation resulted in a significant increase in the explained variance (ΔR^2). For every step the cumulative R^2 is given and ΔR^2 for every additional step. To be able to compare the relative importance of these factors, standardized partial regression coefficients (β) are presented.

To calculate the required sample size we assumed that $R^2 = 0.50$. A value of $R^2 = 0.50$ was decided on for two reasons: (1) Stevens is of the opinion that this is a reasonable estimate for social science research [22]; and (2) anti-HBs titers of the different months were expected to correlate highly and would, as such, explain a large proportion of the variance. According to Stevens [22], given a power of $\beta = 0.80$, and a significance level of $\alpha = 0.05$, six predictors, and $R^2 = 0.50$, at least 70 subjects are required for a meaningful regression equation.

RESULTS

Tables II and III summarize the means, standard deviations, and intercorrelations of the variables under study. Table IV shows the results of the regression analyses.

Table II.—Correlation matrix of dependent and independent variables

	α-HBs-2[a]	α-HBs 6[a]	α-HBs-7[a]	EPCL-0[b]	HSCL-0[b]	EPCL-6[b]	HSCL-6[b]	SI-0[b]	SI-6[b]	Alcohol[c]	Cigarettes[d]	Quetelet
Mean (SD)				52.0 (41.2)	85.7 (21.3)	41.0 (31.8)	84.4 (26.4)	−0.06 (1.81)	−0.01 (1.82)	4.6 (6.4)	0.89 (2.7)	22.1 (2.6)
α-HBs-2	1.00											
α-HBs-6	0.67**	1.00										
α-HBs-7	0.65**	0.52**	1.00									
EPCL-0	0.04	0.02	0.01	1.00								
HSCL-0	−0.24	−0.05	−0.17	0.59**	1.00							
EPCL-6	−0.04	0.02	0.13	0.52**	0.59**	1.00						
HSCL-6	−0.15	−0.12	−0.10	0.56**	0.83**	0.62**	1.00					
SI-0	−0.09	−0.02	−0.09	0.90**	0.88**	0.62**	0.78**	1.00				
SI-6	−0.11	−0.06	0.01	0.60**	0.79**	0.90**	0.90**	0.78**	1.00			
Alcohol	−0.02	−0.26*	0.11	0.18	−0.16	−0.11	−0.12	−0.17	−0.13	1.00		
Cigarettes	0.04	−0.24*	0.09	0.06	−0.03	−0.06	−0.03	0.06	−0.05	0.47**	1.00	
Quetelet	0.30*	−0.09	−0.02	−0.10	−0.20*	−0.13	0.01	−0.17	−0.07	0.02	−0.01	1.00

[a] Anti-HBs levels at months 2, 6, and 7 respectively; [b] scores on the EPCL, HSCL, and SI at months 0 and 6, respectively; [c] drinks per week; [d] number per day.

*$p < 0.05$; **$p < 0.01$.

Table III. – Number of responders (anti-HBs ⩾1 IU/l) and geometric means (GM) with 95% confidence interval (95% CI) of anti-HBs levels in 68 healthy volunteers

	Number of responders	GM (95% CI) responders
Month 2	60	22 (16-30)
Month 6	67	66 (50-87)
Month 7	66	1905 (1318-2754)

Anti-HBs concentrations of the preceding assessment were entered in the first step in the regression analyses of the antibody levels of months 6 and 7. Antibody levels determined at month 2 were found to be highly predictive, accounting for 54% of the variance in antibody concentrations at month 6 (see Table IV). Anti-HBs levels at month 6 were less predictive and accounted for 27% of the variance in antibody concentrations at month 7. Furthermore, gender-related differences were observed in anti-HBs levels at month 6, indicating higher antibody levels for women. In addition, a negative relationship between smoking and anti-HBs levels at month 6 was found. Antibody formations 2 and 7 months after the first vaccination were not significantly influenced by smoking, alcohol consumption, Quetelet index, or gender. In the last step, the SI scores were entered. Regression analyses did not reveal a relationship between stress index score SI-0 and antibody levels at month 2, 6, or 7 after having controlled for the variables entered in steps 1 and 2. Subsequently, SI-6 instead of SI-0 was entered in the statistical analyses of antibody levels at months 6 and 7, and failed to add significantly to the explained variance of any dependent measure as well (see Table IV).

Table IV. – Results of stepwise regression analyses of anti-HBs levels (IU/l) after vaccination

	Anti-HBs month 2			Anti-HBs month 6			Anti-HBS month 7		
	β	R^2	ΔR^2	β	R^2	ΔR^2	β	R^2	ΔR^2
Step 1									
Anti-HBs-2	–	–		0.73***	0.54***		–	–	
Anti-HBs-6	–	–		–	–		0.52***	0.27***	
Step 2									
Gender	0.01			0.24***			0.06		
Quetelet	0.25*	0.07		−0.08	0.66***	0.12***	0.08	0.34***	0.08
Smoking	0.11			−0.18**			0.16		
Alcohol	−0.02			0.03			0.18		
Step 3a									
SI-0	−0.09	0.08	0.01	0.03	0.66***	0.00	−0.11	0.35***	0.01
Step 3b									
SI-6	–	–		0.02	0.66***	0.00	0.08	0.35***	0.01

Gender: 0 = male; 1 = female.
*$p < 0.10$; **$p < 0.05$; ***$p < 0.01$.

Sample size

The nonsignificant associations found may be due to the sample size being too small. *A priori*, the sample size required for a reliable regression equation was calculated assuming that $R^2 = 0.50$ (see Method section: Statistical Analyses). Given the results presented in Table IV, we assessed the minimal number of subjects required to get a significant increment in proportion of explained variance by SI-0 and SI-6 [23]. For the equation with anti-HBs titers at month 2 and month 7 as the dependent variables, 358 and 259 subjects would be required, respectively. No sample size was calculated for the equation with antibody levels at month 6, given the minimal increase in explained variance by SI-0 and SI-6. However, the question arises whether an increase in sample size to get a statistically significant effect is relevant, given the low β and ΔR^2 values, indicating the absence of any clinically relevant effect.

DISCUSSION

The present study was undertaken to evaluate the role of the antigen dose in the previously reported relationship between psychological stress and early antibody response in human volunteers following hepatitis B vaccination [13]. It is well established that, after the primary administration of antigen, antibodies develop slowly. Depending on the antigen dose, antibody titers usually are low or even nondetectable after one vaccination. Due to memory cell formation, re-exposure to the vaccine at a later stage results in a considerable increase in specific antibodies. The anamnestic antibody response reflects the outcome of a complicated series of cellular events triggered by the primary vaccination. Based on the results of our previous study [13] we generated the hypothesis that psychological stress influences, in particular, the initial phase of the immune response. It was envisaged that the degree of stress-mediated immunomodulation in this early stage of immune reactivity could be dependent on antigen dose. In the present study, using the normal and thus higher dose of vaccine, no significant effects of (chronic) psychological stress on anti-HBs antibody levels could be demonstrated.

It has been reported by Moynihan *et al.* [14] that mice, exposed to a single stress session (electric footshock) 24 hours after immunization with a protein antigen, show a reduced anamnestic antibody response compared to nonstressed animals. This result was obtained using low, suboptimal concentrations of the antigen in priming the mice, as well as in the booster injection. Interestingly enough, the suppression of the secondary antibody response was no longer demonstrable using a fivefold higher antigen dose to elicit the anamnestic response. Due to practical constraints we could not include, in the present study, a sample of subjects who received a suboptimal dose of HBs vaccine in the primary vaccination, and were subsequently boostered with the optimal dose of antigen. Therefore, it remains an open question whether the antigen dose-dependent stress-related suppression of the anamnestic HBs antibody response in the human model predominantly reflects a stress-mediated modulation of the induction phase of the immune response. However, based on these results, it is tempting to conclude that under certain conditions stress-induced immunomodulation, in this case antibody formation after vaccination, is influenced by antigen dose.

Until now, studies on the psychological influences on antibody formation after hepatitis B vaccination have not allowed straightforward conclusions, because results

have been overly inconsistent. A recent study [24] showed that disclosure of traumatic experiences in four consecutive writing sessions, prior to vaccination, influences antibody formation in a positive way. Three other studies have also focused on psychological stress. Two of these used the same vaccine and vaccination protocol [11, 12], whereas in the third study some slight modifications had been implemented [13]. The results obtained ranged from negative to positive and nonsignificant correlations between anti-HBs levels and psychological stress measures. The inconsistency in results might at least partly be explained by the fact that in human studies the definition and quantification of psychological stress is complicated. In a recent meta-analysis of the literature on stress and immunity in humans, Herbert and Cohen [25] conclude that objective stressful events are related to larger immune changes than subjective self-reports of stress. When using questionnaires, it is often difficult to control for potentially confounding variables, such as the willingness to reveal stressful events, emotional expression, or defensive coping styles, which have been reported to influence both the way questionnaires are completed and immunity [26, 27]. In addition, psychological variables might exert different influences in the various stages of the immunization process, which in the case of hepatitis B vaccination takes several months. Related to this is the problem that distress levels or other psychological variables might fluctuate during this period. These are not necessarily measured by the questionnaires used or linked to the stressor the subject is supposed to be exposed to.

The paradigm used in this study differs from the one generally employed in human PNI research. In most studies, *in vitro* immune parameters are assessed as measures for *in vivo* immune functioning. It is hypothesized that the neural and endocrine system have exerted their influence on the immune cells prior to blood drawing and in an indirect way are reflected in *in vitro* assays. However, the intricate interplay between the involved systems (neural, endocrine, and immune) *during* an immunological reaction is intrinsically neglected. The level of antibodies reflects the outcome of many steps in the immune response from the initial processing of antigens by antigen-presenting cells to the final production of immunoglobulins by activated B cells. The actions of the integrated system are reflected in the total functioning of the immune system.

We consider the vaccination model as a useful one in human PNI research, but recommend some modifications in future studies. In light of our previous results, the present findings lead us to the suggestion of administering vaccines in suboptimal doses. Furthermore, to diminish the influence of unexpected stressful situations as much as possible, vaccines should be administered with a shorter immunization regimen.

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