The relationship between distress and the development of a primary immune response to a novel antigen

Alison Smith, a,b,* Ute Vollmer-Conna, b Barbara Bennett, b Denis Wakefield, c Ian Hickie, b and Andrew Lloyd c

a School of Psychology, University of Western Sydney, Locked Bag 1797, Penrith South, DC NSW 1797, Australia
b School of Psychiatry, University of NSW, Sydney 2052, Australia
c Inflammation Research Unit, School of Medical Sciences, University of NSW, Sydney 2052, Australia

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Abstract

Forty-five medical students were recruited to examine the effects of distress on the development of an immune response to the novel antigen, keyhole limpet hemocyanin (KLH). The subjects’ level of distress was manipulated by immunizing them either at the time of an important viva voce examination (n = 22) or during examination-free term time (n = 23). This manipulation increased variance amongst the subjects, but the emphasis in this research was on individual distress as a predictor of immune function. In the group as a whole, the likelihood of developing DTH skin responses to KLH was reduced in the more distressed subjects (r = −.45; p = .002), independently of a number of behavioral (e.g., sleep disturbance) and demographic (e.g., sex) variables. Proliferation of T cells against KLH in vitro and the development of anti-KLH IgG antibodies were not related to levels of distress. Thus, cellular, rather than humoral, immune responses in vivo appear susceptible to the influence of distress. This immunization model provides the opportunity to further dissect the basis of these stress–immune pathways.

Keywords: Keyhole limpet haemocyanin; Psychoneuroimmunology; Cellular immunity; Humoral immunity; Primary immune response

1. Introduction

Human research in the field of psychoneuroimmunology has profited in recent years from the development of models that are increasingly biologically relevant, potentially of clinical significance, and also permit the functional assessment of immunity (e.g., Glaser et al., 1992; Kiecolt-Glaser et al., 1995; Kiecolt-Glaser et al., 1996). However, much of this research relies upon recall responses to previously encountered infectious agents (for example, Herpes simplex viruses) or is confounded by variable degrees of prior immunity to the immunogen (e.g., influenza vaccine). This study utilized a standardized method of functional assessment of immunity based on the immunization of subjects with the novel antigen, keyhole limpet hemocyanin (KLH). This model may be considered an immunological challenge that broadly models the events occurring upon host exposure to a previously unencountered pathogen. Such a response necessitates processing and presentation of the novel antigens, followed by the expansion of the effector arm of the response and generation of immunological memory.

KLH is a purified protein derived from an inedible marine mollusc (Megathura crenulata) found off the coast of Southern California (Snyder et al., 1990). The advantages of using KLH in psychoneuroimmunology research is that it allows the comprehensive assessment of an orchestrated cellular and humoral immune response in vivo, it is simple and safe, and it is readily applicable to almost all potential subjects. Both cellular and humoral immunity can be assessed, permitting a comparison of the relative effects of psychological factors on these two arms of the adaptive immune re-
response. In addition, cellular immunity can be assessed in vivo, by delayed-type hypersensitivity (DTH) skin tests (Curtis et al., 1970).

KLH is unlikely to have been seen by the immune system and is thus expected to promote a primary immune response. Consequently, the model should be unaffected by varying levels of prior immunity against the immunogen (as is the case for more naturalistic immunization models such as influenza vaccine). However, past research suggests that some individuals demonstrate responses to KLH prior to immunization with the antigen. In vitro blast transformation of human leucocytes from individuals with no known history of exposure to KLH has been demonstrated (Green and Borella, 1971), although a failure to find this response has also been observed in unimmunized individuals (Burke et al., 1977). In unimmunized human subjects, high avidity IgG antibodies against KLH have been demonstrated using radioimmunoassay (Moroz et al., 1973) and by a sensitive haemagglutination technique (Burke et al., 1977). In unimmunized individuals, the development of DTH responses to an intradermal dose of 100 μg of KLH has also been demonstrated (Hortobagyi et al., 1981), although classical DTH responses are typically elicited by considerably smaller doses of antigen.

These baseline responses are likely to be due to the presence of cross-reacting epitopes, such as those that have been demonstrated with antigens from phylogenetically close species (Amkraut et al., 1969); mammalian red cell stroma (Frick and Shimbor, 1970); the Thomson–Friederich antigen present on bladder and other adenocarcinomas (Wirguin et al., 1995) and a carbohydrate epitope on the surface of Schistosoma mansoni larval schistosomes (Yuesheng et al., 1994). In fact, the high immunogenicity of KLH may in part reflect the existence of these cross-reacting epitopes (Harris and Markl, 1999). Consequently, an important part of the research reported in this paper is an examination of the extent to which subjects display significant responses to KLH prior to immunization.

Before commencement of this research a suitable immunization protocol with an appropriate immunization dose and timing of follow-up assessment was developed. A response rate of approximately 70% was deemed appropriate so that both immune enhancing and immune suppressive effects of distress could be observed. The method and results of this protocol development are reported briefly in this paper, prior to an examination of the effects of distress on anti-KLH immunity. The relationships between the various measures of immune function are also examined, since they may clarify mechanisms underlying psycho-neuro-immunological influences.

The stressor chosen for this study was a medical student examination, a widely used model in psychoneuroimmunology research (reviewed by Kiecolt-Glaser and Glaser, 1992). Medical students provide the advantage of generally low rates of pre-morbid psychological dysfunction. Thus, this paradigm facilitates an evaluation of the effects of a relatively minor and common stressor on anti-KLH immunity in a psychologically and physically healthy group of individuals. It should be noted, however, that the stressfulness of the examination, as well as the individual’s perceived competence in the subject to be examined may vary significantly. Thus, the analyses reported in this paper emphasize the prediction of immunological outcomes by the subjective distress levels recorded for each individual, rather than exclusively by the comparison of study groups (Vedhara and Nott, 1996). Levels of serum noradrenaline and cortisol were also determined. These measures generally correlate with distress (Frankenhaeuser, 1983) and provide covert indices of psychological state. They may also be related to changes in immune function (Black, 1994).

2. Materials and methods

2.1. Protocol development

2.1.1. Subjects

Twenty-one healthy volunteers aged between 18 and 30 years (12 males and nine females) volunteered to take part in the research. Of these, six participated in an examination of the kinetics of the immune response and a further 15 in an examination of the rates of response. These volunteers were university students experiencing normal everyday levels of distress since they were recruited during examination free term time.

The research reported in this paper was approved by the Committee on Experimental Procedures Involving Human Subjects of the University of New South Wales. Informed, written consent was obtained from all subjects before the commencement of procedures. Subjects were excluded from the study if they (i) had a history of seafood allergy; (ii) were pregnant; (iii) had experienced a significant illness or operation in the past month; (iv) had a history of a major immune-related illness (for example, diabetes or cancer); (v) were taking any immuno-suppressive medication or (vi) were symptomatic with an intercurrent infective illness at the commencement of the study.

2.2. Immunization dose

KLH (Pierce, Rockford, USA) was adsorbed to the adjuvant alum (Pierce) according to the manufacturer’s instructions. The immunization protocol investigated (and finally chosen) consisted of 0.1 mg of KLH (1.25 mg/ml) adsorbed to 0.9 mg of alum (45 mg/ml) administered into the deltoid muscle.
2.3. Assessment of anti-KLH immunity

2.3.1. KLH-induced DTH skin responses

Delayed type hypersensitivity reactions were assessed by administration of 0.001 mg of KLH in 0.01 ml of saline solution as an intradermal injection into the volar aspect of the arm. The response was read 48 h after administration by measuring induration in millimetres for two diameters at right angles. The mean of these measurements was recorded. A positive response was defined as a mean diameter of greater than 2 mm (Hortobagyi et al., 1981). Such a classification is regularly used in clinical settings where DTH skin test categories of response are used as an index of health status (Gmüör et al., 1976; Hersh et al., 1971; Poenaru and Christou, 1991). Classification of individuals as responders or non-responders for DTH (and the other measures of anti-KLH immunity) also facilitated an examination of the extent to which individuals demonstrated significant anti-KLH responses at baseline.

2.3.2. KLH stimulation assays

Peripheral blood mononuclear cells (PBMCs) were cryopreserved until the completion of each experiment so that samples collected longitudinally from each subject could be assessed in parallel. The blood collection, as well as freezing and thawing protocols, and proliferation assays were performed under strict endotoxin-minimised conditions. Standard cryopreservation and thawing methodology were employed (Gjerset et al., 1986) with the exception that cells were resuspended in supplemented RPMI 1640 (Flow Laboratories, Sydney, Australia) that contained 20% autologous plasma.

Procedures for the antigen proliferation used standard methods (Maluish and Strong, 1986; Snyder et al., 1993). In brief, for the antigen stimulation assays, aliquots of 100 μl of the cell suspensions were added to quadruplicate wells of round-bottomed microtitre cell culture plates (Nunc, Roskilde, Denmark) in addition to 100 μl of KLH in supplemented RPMI 1640 at final concentrations of 0, 0.01, 0.05, and 0.1 mg/ml. Each plate included samples from all time points of one subject plus cells from a positive control subject (multiply immunized with KLH) to ensure reliability of the assay. The cultures were incubated at 37°C and 5% CO₂ in air for 5 days before measurement of incorporation of tritiated thymidine. Stimulation indices are reported for the highest concentration of KLH in vitro. A stimulation index of greater than 5.0 was deemed a positive response. Results are reported for the highest concentration of KLH in vitro.

2.3.3. Enzyme-linked immunosorbent assay

Sera were frozen in aliquots at −20°C until the completion of the experiment so that all samples from each subject could be included in the same assay. The procedures employed standard ELISA methods (Snyder et al., 1990; Voller and Bidwell, 1986). In brief, ELISA plates (Polysorp, Nunc, Roskilde, Denmark) were coated with 1 μg/ml of KLH in a carbonate buffer (pH 9.6) and kept at 37°C for 2 h and then overnight at 4°C. The plates were blocked with 1% non-fat dry milk powder in PBS (200 μl per well). Sera at concentrations of 1/100 or 1/1000 in blocking buffer plus 0.05% Tween 20 were added to the wells (100 μl per well). The detection antibody (horseradish peroxidase conjugated rabbit anti-human IgG; Dako, Glostrup, Denmark) was added at a concentration of 1/500 in blocking buffer plus 0.05% Tween 20 (100 μl per well). The optical densities (ODs) of the wells were read using an automated plate reader (Multiskan PLUS Mk II, Titertek, Sydney, Australia) with a filter at 405 nm.

Consistent with the widespread adoption of an ELISA OD criterion for seroconversion (as opposed to end-point titers) in clinical practice, seroconversion was defined as an OD level three standard deviations above the mean of six unimmunized control subjects. These negative control sera and a positive control serum were routinely measured with each ELISA run to control for assay-to-assay variability. Results are reported for sera concentrations of 1/100.

2.4. Kinetics and rate of response to KLH

To examine the kinetics of the development of immune responses to KLH, six subjects were immunized with KLH. Blood was drawn for analysis of T-cell proliferation against KLH and assessment of anti-KLH IgG antibody responses at the time of immunization (referred to as “baseline”) and then weekly for four subsequent weeks.

The rate of response to KLH/alum was assessed in a further 15 subjects. The DTH skin test to KLH was measured and blood was drawn for in vitro analyses at immunization and again three weeks later (“follow-up”).

3. The effects of distress on anti-KLH immunity

3.1. Subjects

To evaluate the sensitivity of the KLH model to distress, 45 medical students, aged between 21 and 31 years (mean age = 22.4, SD = 1.85) were allocated to a control (N = 23) or experimental (N = 22) group according to their availability for follow-up assessment. There were 13 males and 10 females in the control group and 10 males and 12 females in the experimental group.

3.2. Measures of distress

The main purpose of immunizing subjects at either a time of low or high stress was to increase variance in the
sample. The assumption underlying this research was that it is the effect of stressor exposure (level of distress) experienced by the participant that determines immune function, rather than just that exposure. Thus, the profile of mood states (POMS) questionnaire was administered to measure current psychological distress. Total POMS scores are presented. Subjects were asked to rate how well 65 individual descriptors of feelings (e.g., “tense,” “blue,” and “exhausted”) applied to them over the past week, including the current day, on a five-point rating scale from 0 (“not at all”) to 4 (“extremely”). The reliability and construct validity of this scale have been demonstrated (McNair et al., 1981).

3.3. Behavioral assessment

Behavioral factors known to affect immune function were also considered (Kiecolt-Glaser and Glaser, 1988; Palmblad, 1981). Subjects were asked to report the number of cigarettes smoked daily, their average weekly alcohol consumption, amount of aerobic exercise, and sleep changes in the past week and any weight loss over the previous month. Female students recorded the stage of their menstrual cycle. The exclusion criteria ensured that participants with either acute or chronic illnesses, or taking immuno-suppressive medication were not included in the study.

3.4. Assessment of anti-KLH immunity

The procedures outlined for the protocol development stage were followed, with the exception that a further concentration of KLH (0.25 mg/ml) was added to the in vitro T-cell proliferation assays.

3.5. Hormonal assays

Sera for use in the cortisol and noradrenaline assays were stored at –70°C. Serum cortisol levels were determined by radioimmunoassay using commercially available kits (Orion Diagnostica, Espoo, Finland) by the Department of Endocrinology, The Prince of Wales Hospital, Sydney. Noradrenaline assays were conducted using high-performance liquid chromatography by the Department of Clinical Chemistry, The Prince of Wales Hospital, Sydney.

3.6. Experimental protocol

The weight of evidence from animal models suggests that antigen encounter must coincide with, or follow shortly after the stressor (within approximately 24 h), if immuno-modulatory effects are to be observed (Blecha and Kelley, 1981; Cocke et al., 1993; Coe et al., 1987; Fleshner et al., 1992; Hibma and Griffin, 1994; Irwin, 1993; Okimura and Nigo, 1986; Wood et al., 1993). Consequently, experimental subjects were immunized within 15 min of the completion of a yearly viva voce examination, which is widely regarded by the students as being stressful. Control subjects were immunized in normal examination-free term time.

Prior to the immunization, an intra-dermal dose of KLH was administered to evaluate DTH responsiveness and blood was drawn for in vitro assays, as well as cortisol and noradrenaline assays. Demographic information was collected and the self-report instruments were completed. Three weeks later, DTH skin test responses to KLH were recorded again and blood was drawn for the in vitro anti-KLH measures. Responses to the distress and behavioral questionnaires were recorded.

As the examination schedule constrained the timing of immunization, experimental subjects were stratified as being immunized in the morning (before 1:00 p.m.; \( n = 10 \)) or afternoon (after 1:00 p.m.; \( n = 12 \)) with control subjects immunized either in the morning (\( n = 14 \)) or afternoon (\( n = 9 \)). The stratification designated by time of collection was maintained for case-control pairs in the KLH stimulation assays.

3.7. Statistical analysis

Comparisons of the development of anti-KLH immunity between the control and experimental groups were performed using \( t \) tests or the \( \chi^2 \) statistic (for categorical data). The prediction of immune responses from individual factors was performed using point biserial correlations. The influence of group membership and behavioral factors on any observed relationships was assessed using direct logistic regression.

4. Results

4.1. Protocol development

The immunization procedure was well-tolerated by all subjects. The injection produced minimal, transient discomfort in all but one of 21 subjects who reported local tenderness, which lasted for three days. Proliferative responses and an anti-KLH IgG antibody response were evident in all but one subject by week 3. Based on these data, it was decided to assess immune function at 3 weeks post-immunization in subsequent experiments.

The response rate using the specified dose of KLH adsorbed to alum was 73% for both the development of anti-KLH IgG responses and DTH skin test responses against KLH. Eighty percent of subjects developed proliferation responses in vitro of T cells against KLH. These response rates were deemed appropriate to allow an examination of the immune enhancing and suppressive effects of psychological variables. In addition, it was
anticipated that the response rate would not be so great as to mask the immuno-modulatory effects of these variables (Vedhara and Nott, 1996).

4.2. The effects of distress on anti-KLH immunity

4.2.1. Group comparisons

POMS. At baseline, subjects in the experimental group \( M = 60.1, \) SD = 42.9) displayed greater negative mood than the control group \( M = 26.7, \) SD = 29.3) \( t(43) = 3.1; p = .004 \). At follow-up, there were also significant differences between the control \( M = 37.8, \) SD = 37.6) and experimental \( M = 6.9, \) SD = 25.2) subjects on the total POMS score, with the control subjects reporting higher levels of negative mood than the experimental subjects \( t(43) = 3.2; p = .002 \).

DTH responses to KLH. At baseline, no subject displayed a DTH response. Of 45 subjects, 41 reported zero induration. At follow-up, the mean induration in the control group \( n = 23 \) was 5.6 mm (SD = 4.6) while in the experimental group \( n = 21 \) the mean response was 5.4 (SD = 7.8). This difference was not significant \( t(42) = .088, p = .93 \). The pattern of responses at follow-up is reported in Fig. 1 at which time 68% (30 of 44) subjects developed DTH responses. Significantly more control subjects (19 of 23) developed these responses to KLH than experimental subjects (11 of 21) \( \chi^2(1, N = 44) = 4.62, p = .03 \).

Lymphocyte proliferation against KLH. At baseline the mean stimulation index for the control group \( n = 23 \) was 3.8 (SD = 1.5) and for the experimental group \( n = 22 \) it was 3.3 (SD = 1.0). This difference was not significant \( t(43) = .717, p = .48 \). At follow-up, the mean stimulation index for the control group was 5.6 (SD = 3.5) and for the experimental group \( n = 22 \) the mean index was 8.1 (SD = 6.1). These differences were not significant \( t(43) = .79, p = .44 \). The proliferation responses are presented in Figs. 2 and 3. Considerable variability of responses is evident, both at baseline and follow-up. At baseline, seven subjects (five control and two experimental) had stimulation indices greater than 5. At follow-up, 56% (25 of 45) subjects developed proliferation responses against KLH. The number of subjects who developed a proliferative response in the control \( n = 13 \) and experimental \( n = 15 \) groups did not differ \( \chi^2(1, N = 45) = 1.14, p = .29 \).

Anti-KLH IgG antibodies. The mean optical densities in the ELISA at baseline for the control group \( n = 23 \) was 0.208 (SD = 0.14) and for the experimental group \( n = 22 \) the value was 0.188 (SD = 0.20). At follow-up the mean for the control group \( n = 23 \) was 1.061 (SD = 0.88) and for the experimental group \( n = 23 \) it was 1.151 (SD = 0.82). These differences were not significant at baseline \( t(43) = .396, p = .69 \) or at follow-up \( t(43) = .357, p = .72 \). The variability of responses at
baseline and follow-up is illustrated in Figs. 4 and 5. One subject was seropositive at baseline, although, this subject did not have a proliferation or DTH response at that time. In total, 56% (25 of 45) of subjects developed anti-KLH IgG responses. The number of subjects developing antibody responses did not differ between the control \((n = 13)\) and experimental \((n = 12)\) groups \(\chi^2(1, N = 45) = 0.02, p = .89\).

### 4.2.2. Prediction of immune function from individual difference factors

**POMS.** Table 1 provides correlations between the POMS scales (at baseline and follow-up) and the indices of anti-KLH immunity at follow-up. The POMS score at follow-up is included since there is evidence in animal models that psychological state at the time of immune assessment may influence immune competence (Dhabhar and McEwen, 1996). Distress at baseline and the likelihood of developing a DTH skin test response three weeks later were negatively correlated \((r = -0.45, p = .002)\). There were no correlations between psychological state at baseline and proliferation against KLH in vitro \((r = 0.02, p = .90)\) or the development of antibody responses \((r = 0.04, p = .79)\).

**Hormonal measures.** The relationship between levels of noradrenaline at baseline and the development of cutaneous DTH against KLH three weeks later was examined using a point biserial correlation. The correlation between noradrenaline levels and the POMS scores at baseline was .29 \((p = .05)\). There were no other significant correlations between the physiological indices of distress (levels of noradrenaline and cortisol) at baseline and the development of anti-KLH 3 weeks later (Table 2).

**Behavioral and demographic measures.** For both the in vitro proliferation of T cells against KLH and the development of anti-KLH IgG antibodies, there was only one significant association with the behavioral and demographic measures. KLH-stimulated proliferation responses were more likely to develop in females \((n = 16)\) than in males \((n = 9)\) \(\chi^2(1, N = 45) = 5.14, p = .02\). There were no correlations between the DTH skin test responses against KLH and continuous behavioral variables (Table 3). DTH responses were not related to sleep disturbance at baseline \([\chi^2(1, N = 44) = 0.47, p = .66, \text{Fisher’s exact test}]\) or at follow-up \([\chi^2(1, N = 44) = 0.09, p = 1.0, \text{Fisher’s exact test}]\). The subject’s sex \([\chi^2(1, N = 44) = 0.00, p = 1.00]\) and racial group membership \([\chi^2(1, N = 43) = 0.01, p = .95]\) were unrelated to the development of DTH skin test responses against KLH.

**Analyses with subjects positive to KLH at baseline removed.** Seven subjects displayed proliferative responses against KLH and one demonstrated significant anti-KLH IgG levels at baseline. The major results of the present study were re-analyzed without these 8 subjects included leaving a total of 37 subjects (18 control and 19 experimental).

Again, there were no significant differences between the control \((n = 18)\) and experimental \((n = 19)\) subjects in the development of anti-KLH IgG \([\chi^2(1, N = 37) = 0.04, p = .89]\).
or in control and experimental subjects in the proliferation of T cells against KLH in vitro $[\chi^2(1, N = 37) = 2.17, p = .14]$ at follow-up. With this smaller number of subjects, the differences between control and experimental subjects who demonstrated DTH responses against KLH at follow-up was significant $[\chi^2(1, N = 36) = 4.50, p = .03]$.

There was no relationship between the POMS at baseline and the levels of anti-KLH antibodies ($r = .13, p = .47$) or T-cell proliferation ($r = .14, p = .42$) at follow-up. The correlation between these POMS scores at baseline and cutaneous DTH at follow-up was .40 ($p = .02$).

### Logistic regression

The possibility that the relationship between distress and DTH skin test responses was actually accounted for by behavioral/demographic factors was further explored using direct logistic regression. Data was available for 45 subjects. Analyses were performed using SPSS 9.0.

Group status was entered in the logistic regression equation to control for differential treatment of the subjects in the control and experimental groups. To reduce the number of variables included in the logistic regression, only behavioral or demographic variables related to the immune measure at the .1 level of significance were considered for inclusion in the model.
Levels of aerobic exercise at baseline and alcohol consumption at follow-up were significant at this criterion level. However, only 4.4% (2) of subjects engaged in more than 5 h of aerobic exercise per week so this variable was not considered further. Alcohol consumption varied considerably ($M = 4.2, SD = 10.2$) at follow-up and has been associated with a decrement in immune function (Glassman et al., 1985; Roselle and Mendenhall, 1982; Saxena et al., 1986; Stacey, 1984) and so was included in the logistic regression analysis.

The model was significant [$\chi^2(3, N = 44) = 12.35$, $p = .01$] and correctly classified 93% of responders and 50% of non-responders (80% overall). A model without group status included was also considered. This model was not significantly different from the model that included group status [$\chi^2(1, N = 44) = 0.21$, $p > .05$]. This model correctly identified 93% of responders and 57% of non-responders. The Wald statistic was significant for the POMS ($z = 7.08$, $p = .01$), but alcohol consumption at follow-up was not a significant predictor of the outcome ($z = 1.31$, $p = .25$). However, removal of alcohol from the regression equation reduced the number of correctly classified cases to 75%.

4.2.3. Relationships between immune measures

There were no significant associations in the likelihood of responding to KLH between the two measures of cellular immune function [$\chi^2(1, N = 44) = .17$, $p = .68$], between anti-KLH IgG levels and KLH-stimulated T-cell proliferation [$\chi^2(1, N = 45) = 0.00$, $p = .95$] or between the IgG levels and DTH skin test responses against KLH [$\chi^2(1, N = 44) = 0.00$, $p = .98$]. The correlation between pre- and post-immunization KLH-stimulated proliferation in vitro was .35 ($p = .02$) and between pre- and post-anti-KLH antibody responses it was .53 ($p = .00$).

5. Discussion

The experimental manipulation was successful in increasing variability in distress amongst the subjects at baseline. In addition, at follow-up, the experimental, but not the control, subjects had commenced the end-of-semester vacations and consequently were considerably less distressed than the control subjects. This was not an ideal experimental design, but it did have the advantage of allowing assessment of a range of distress measures (very low to high) at different stages in the development of an immune response. Individuals who were exposed to an examination were less likely to develop DTH skin test responses against KLH, although the other measures of anti-KLH immunity were not affected.

However, the emphasis in this research was on the capacity of individual factors to predict anti-KLH immunity, rather than on group differences. Psychological distress at immunization affected the in vivo cellular immune measure (DTH) rather than in vitro T-cell proliferation or antibody production against KLH at follow-up. This influence on DTH was independent of behavioral and demographic factors (Table 3), although the role of alcohol consumption at follow-up requires further investigation. The reduction in DTH skin test responses against KLH suggests that there was a deficit in production of antigen-specific activated and memory T cells at baseline that initiate the DTH response three weeks after immunization (Roitt et al., 1998). There was no evidence of an effect of psychological variables at the time of follow-up on anti-KLH immunity (Table 1).

The development of DTH skin test responses against KLH in vivo and KLH-stimulated T-cell proliferation in vitro both depend on the presence of memory and/or effector T cells specific for KLH and may therefore be expected to be correlated. However, the lack of a relationship between the two indices of cellular immunity has been demonstrated previously (Curtis et al., 1970) and may reflect the different compartments of the immune system (skin versus blood) and the different APCs (principally Langerhans cells in DTH versus monocytes and B cells in the in vitro stimulation) involved in these measures.

The failure to find a relationship between the cellular and humoral arms of the immune response against KLH may reflect differences in T-cell and B-cell epitopes. This possibility is supported by the observation that subjects positive for T-cell proliferation at baseline (probably as a result of cross-reacting epitopes) were not also positive for the development of anti-KLH IgG antibodies. In addition, the concept of a divergence of DTH and humoral immunity is recognised. Notably, the strict definition of the term anergy is that it is a deficit in the formation of DTH in an individual with an intact antibody response (Dwyer, 1984). In addition, a dose-dependent, inverse relationship between DTH and vigorous antibody responses (immune deviation) has been observed (Parish, 1971; Parish, 1996; Parish and Liew, 1972). Immune deviation has been recently re-interpreted in terms of genetically or environmentally determined individual differences in the propensity to develop Th1 and Th2 responses. A number of host and antigen factors influence this propensity (Seder and Mosmann, 1999). Alternatively, it may be that correlations between anti-KLH antibodies at follow-up and levels of distress at the time of immunization may have been detected if the kinetics of antibody production was examined over each of the three weeks post-immunization.

Noradrenaline (but not cortisol) levels at baseline were correlated with the likelihood of developing DTH against KLH at follow-up. In addition, these hormone levels were correlated with levels of distress at baseline, suggesting a mechanism by which mood may affect...
immune function in this sample. These findings are particularly interesting given that it was not possible to exercise complete control over the time of day that the subjects were immunized, resulting in a potential influence on the results of diurnal variations in hormonal levels (Kågedal and Goldstein, 1988; Rosenfeld et al., 1971). Furthermore, the lack of a correlation between DTH and cortisol levels may have been due to the occurrence of threshold effects rather than a linear relationship between the variables (Coe et al., 1987). Neither noradrenaline nor cortisol levels at baseline were correlated with KLH stimulated proliferation or antibody responses.

Perhaps the most interesting question arising from this research is the extent to which alterations in DTH in response to distress reflect an immunological disturbance of clinical significance. Cutaneous DTH is simply a convenient representation of systemic hypersensitivity in vivo (Dwyer, 1984) and the likelihood that reductions in DTH against KLH may be clinically significant is suggested on a number of grounds. DTH skin tests are widely used as an indication of the capacity to induce cell-mediated immunity, and hence relative protection against tuberculosis (Beck, 1991). These responses have been of prognostic significance (Dwyer, 1984; Hortobágyi et al., 1981; McLean, 1988; Poenaru and Christou, 1991), although DTH skin test responses are not always predictive of the outcome of disease states (Golub et al., 1974). Overall, these data emphasise the value of including an in vivo measure of immune function in PNI research (Hickie et al., 1990), particularly when the focus of the research is on the clinical significance of alterations in immune status in response to psychological perturbations (Vedhara et al., 1999).

The variance accounted for by negative affect at the time of immunization was 18% (Table 1). Thus, the magnitude of the effect of distress on the development of DTH skin test responses to KLH in this sample of healthy, young students was not large. With more profound or prolonged levels of distress, or when individuals are already immuno-compromised (such as in the elderly or medically ill) the effect is likely to be more significant (Cohen et al., 1998). Furthermore, the sample used in this study was relatively homogeneous medically, psychologically and behaviorally. This homogeneity may have acted against the capacity to detect distress/immune relationships.

Assessing the level of pre-immunization anti-KLH immunity is important in determining whether a primary immune response is being observed at follow-up. Pre-immunization responses to a truly novel antigen are not expected. However, seven subjects demonstrated significant proliferation responses against KLH at baseline and another subject demonstrated significant levels of anti-KLH antibodies at baseline. There was also considerable variability in the immune responses of subjects at baseline (Figs. 2 and 4). These data may well account for the correlations between pre- and post-immunization KLH-stimulated proliferation in vitro ($r = .35; p = .02$) and anti-KLH antibody responses ($r = .53; p = .00$) by way of a quasi-secondary immune response. One means by which to deal with these results in psychoneuroimmunology research would be to eliminate from analyses those subjects developing baseline responses. In the present study, this procedure produced results consistent with the larger group findings, indicating that baseline levels of response had little effect on overall conclusions. However, in research examining mechanisms of a primary immune response removal from analyses of subjects positive at baseline would be essential. The stimulation index adopted in the research reported here was a very conservative one since the aim was to detect biologically significant effects of distress on anti-KLH immunity. More liberal indices may be appropriate with other research questions.

In conclusion, immunization of individuals with KLH provides information about the capacity to induce an immune response in vivo. Cellular rather than humoral immune responses were affected by distress levels and the effect may have been mediated by noradrenaline. The occurrence of distress at the time of sensitization rather than elicitation appears to bring about these alterations in immune competence. Further studies to elucidate the cellular and molecular basis of this distress-induced reduction in immunity in humans are warranted.

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