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Keyhole limpet haemocyanin - a model antigen for human immunotoxicological studies

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bcp.12422

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Summary

Immunisation with a T-cell dependent antigen has been promoted as a reliable and sensitive tool for assessing the influence of putative immunotoxic exposures or agents on immune function. Keyhole limpet haemocyanin (KLH) is a very large, copper-containing protein molecule derived from the haemolymph of the inedible mollusc, *Megathura crenulata*. KLH is a highly immunogenic T-cell dependent antigen that is used increasingly in immunotoxicological studies, particularly in those involving animals. This report systematically reviews the human clinical studies that have used trans-cutaneous KLH immunisation for assessment of the influence of various physiological and disease states and exposures on immune function over the last twenty years (1994 – 2013). These studies varied in their immunisation protocols, formulation of KLH, dose, site and route of administration and immunoassay platforms developed to assess KLH-specific responses. KLH immunisation has been well tolerated with only mild to moderate adverse effects reported. Though very promising as a model antigen candidate in immunotoxicology research, more work on standardising immunisation and immunoassay protocols is required.

Introduction

The effect of extrinsic (e.g. environmental exposure) or intrinsic (e.g. psychological distress) factors on the human immune system can be effectively assessed by quantifying the antigen-specific response to immunisation with a T-cell dependent antigen, although care needs to be taken with the choice of antigen (1,2). Keyhole limpet haemocyanin (KLH) is an immunogenic protein antigen that is xenogeneic to the mammalian immune system. It is used primarily in animal immunotoxicological studies but has a number of applications in the human context including as a vaccine conjugate peptide and in immunotherapy. However, practical aspects regarding the utility of KLH as a diagnostic antigen in human immunotoxicology studies have not previously been reviewed in detail. This paper describes the ideal attributes of a vaccine candidate for human immunotoxicology studies and the structure and immunostimulatory properties of KLH. We then present a systematic review of the use of KLH immunisation via trans-cutaneous routes in human immunotoxicological studies over the period 1994-2013, including its safety profile and the relevant immunoassay platforms required to assess the immune response to immunisation.

Use of T-cell dependent antigens in immunotoxicological studies

Quantification of the primary antibody response to immunisation with a T-cell dependent (TD) antigen (e.g. sheep red blood cells, ovalbumin, KLH, tetanus toxoid, hepatitis B surface antigen) is a sensitive method for assessing immunocompetence (3–6). The immune response to immunisation with a TD antigen is commonly referred to as a T-cell dependent antibody response (TDAR) (7).

Immunisation with a TD antigen permits assessment of the complex primary immune response that involves antigen presentation, priming and collaboration of T and B lymphocytes, antibody production and cytokine-dependent antibody class switching (6).

In animal immunotoxicological research, assessment of a TDAR using sheep erythrocytes or KLH has become the functional immune assay of choice (8–10). For human immunotoxicology studies, opportunistic monitoring of the responses to routine childhood vaccinations (e.g. tetanus, diphtheria, pertussis) has been advocated (11,12).

What makes an ideal immunisation antigen candidate for immunotoxicology studies?

The properties of an ideal TD antigen for immunisation have been previously described (11,13,14) and include the following:

i) Pure homogeneous substance available as a clinical grade product;

ii) Harmless, if not beneficial, to the recipient;

iii) Highly immunogenic for the entire population without any genetic restriction;

iv) Have no cross-reacting antibody;

v) Elicit predictable primary immune responses (without need for an adjuvant) following a single administration;

vi) Produce a measurable immune response that can differentiate subtle changes in immunomodulation (i.e. have high sensitivity to detect change) using validated immune assays.

Commercially available vaccines (e.g. hepatitis B, influenza, tetanus) have the advantages of already having passed strict safety regulatory processes, providing a protective benefit for study participants and being available in a clinical grade formulation. The main disadvantages are that in a non-paediatric population, many participants will have been exposed to antigen from wild-type infection or previous vaccination. Furthermore, commercial vaccines produce a robust immune response that potentially overwhelms the assay's ability to detect subtle changes in immune response. As

mentioned, KLH is often used in animal immunotoxicological research and has many of the qualities of an 'ideal' vaccine candidate (13).

KLH: Structure, properties and biological uses

KLH is derived from the haemolymph of the inedible marine mollusc, *Megathura crenulata*, native to the Pacific coastal waters of California and Mexico (15). KLH is traditionally harvested from molluscs by lethal ex-sanguination, leading to concerns regarding the sustainable supply of research and commercial KLH quantities given the depletion of native marine stocks. However, new non-lethal techniques for the extraction of haemolymph and sustainable aquaculture practices have lessened this concern (16).

Hemocyanins are cylindrical, copper-containing molecules that act as oxygen-transporting proteins for many mollusc species. KLH is an extremely large molecule (~8,000kDa) comprising a variable number of sub-units (KLH1 (390kDa) and KLH2 (350kDa)) (15,17). The remarkable immunostimulatory properties of KLH result from high antigenicity derived from numerous carbohydrate and peptide epitopes (15,18).

The potent immunogenicity of KLH has been known for over 40 years (19–21) and since that time KLH has been used extensively in animal and human research to delineate cellular and humoral immune responses, as a carrier protein for cancer vaccines and as bladder cancer immunotherapy (22,23). KLH appears to have anti-proliferative action against certain tumour cell lines, including breast, pancreatic and oesophageal cancer (24,25).

KLH is xenogeneic to the human immune system and therefore promotes a reliable primary immune response, however individuals with exposure to the fluke *Schistosoma mansoni* can have cross-reactive antibodies to a shared carbohydrate epitope (18).

KLH immunisation as a test of immune status in humans: a systematic review

OBJECTIVES

KLH immunisation has been used in a number of clinical studies to assess the influence of various physiological and disease states and exposures on immune function. These have included psychological states (26–30), cardiovascular exercise (31,32), cancer and/or chemotherapy (13,21,33,34), immunodeficiency states (35–37), atopy and asthma(38,39) and autoimmune disease(40,41). Our objectives in this systematic review were to: i. Survey the formulations, modes of administration and doses used; ii. Assess the available safety data; and iii. Summarise the measures of antigen-specific immune response used, both antibody- and cell-mediated.

METHODS

We identified studies conducted over the last 20 years where a trans-cutaneous KLH immunisation route was used for the purpose of assessing immune function and where KLH-specific immune parameters were the primary or secondary immunological end-points. Studies were identified from PubMed, US Library of Medicine (http://www.ncbi.nlm.nih.gov/pubmed) using the following parameters – Keywords: "KLH" or "keyhole limpet"; Time period: 1 January 1994 to 31 December 2013; Language: English. Only studies with online abstracts available were screened for possible inclusion in this review. Table 1 details the sixteen human clinical trials found.

[See Table 1]

RESULTS

1. **Formulation, administration and dose**

KLH for clinical use comes in two forms – high molecular weight (HMW) and sub-unit preparations. Both preparations are available in a clinical grade formulation that is sterile and endotoxin and pyrogen free. Sub-unit KLH (~400kDa) is often used as a vaccine carrier protein that is coupled to a carbohydrate or other non-immunogenic molecule to boost T-cell priming (e.g. novel anti-cancer vaccines (46)). HMW KLH (or 'native' KLH) preserves the weight of the larger molecule, although manufacturers have found quality control issues to be more challenging (47).

HMW-KLH has greater immunogenicity compared with sub-unit KLH, as demonstrated in the study conducted by Miller et al (33). Here, three forms of 1000µg KLH were administered to healthy participants: HMW KLH, sub-unit KLH or sub-unit KLH with mineral oil adjuvant (Montanide ISA-51). A similar and potent immune response was seen in participants immunised with the HMW-KLH and sub-unit KLH with adjuvant, but not in those immunised with sub-unit KLH alone. It was postulated that the lack of response was not due to a lack of important immunogenic epitopes in sub-unit-KLH but, instead, was due to an adjuvant property of HMW KLH that was successfully substituted by use of the mineral oil adjuvant.

A measureable, robust antigen-specific immune response can be generated following administration of KLH via a number of routes – intra-dermal (35,37,40), sub-cutaneous (33,36,43–45), intra-muscular (28,30–32,38,42) and inhalational (39). Studies that administered KLH intra-muscularly used the deltoid muscle. Site of sub-cutaneous administration was documented over the deltoid (44) or "arm" (45), whilst intra-dermal immunisation was given in the upper arm (35) or forearm (40). In those studies assessing DTH response, intra-dermal KLH was administered to the volar aspect of the forearm (29), upper arm (13) or gluteal aspect of the leg (44).

Older studies have used immunisation doses of KLH up to 5000µg (21), although the range in the recent clinical studies reviewed was 8 µg to 1000 µg, with 100 µg being the most frequent dose. Only one study reviewed was unable to detect a quantifiable antibody response post-immunisation (34), which was likely attributable to the immunosuppressed state of the study population. In the

only published study to assess the effect of different doses of the same KLH formulation, Curtis et al (21) reported no significant difference in the kinetics or magnitude of the immune response amongst participants immunised with 10µg, 100µg or 5000µg of HMW KLH.

2. Safety profile

KLH has an excellent clinical safety profile, as noted by several authors (23,29). In their comprehensive review, Harris & Markl (15) state, "Importantly, KLH is considered to be an extremely safe substance for *in vivo* use in man, as a direct antigenic stimulus and immunotherapeutic agent" (*pp.* 614). KLH immunotherapy for bladder cancer has received European regulatory approval (48). There was no report of significant adverse events related to the use of KLH in any human clinical study reviewed for this paper. Reports of mild adverse effects (e.g. itching, rash, soreness at injection site and malaise) attributable to KLH vaccination occurred in 9 of 103 participants in one clinical trial (36). Importantly, potential adverse effects increase if vaccine adjuvants are used in conjunction with KLH (e.g. alum, oil-water adjuvants or mineral-oil adjuvants)(49,50).

Despite the excellent safety profile of KLH in clinical studies, most studies have excluded patients with a history of shellfish allergy. There have been reports of anaphylaxis following ingestion of a related mollusc species, the grand keyhole limpet (GKL), with cross-antigenicity between GKL, abalone and KLH demonstrated (51).

3. Measures of antigen-specific immune response

Quantifying the KLH-specific antibody response

Most studies that have used KLH immunisation to assess an antigen-specific immune response have measured KLH antibodies (14 of the 16 unique studies listed in Table 1), with most studies utilising indirect ELISA assays. A multiplex flow cytometric bead array platform has been recently reported that allows a semi-quantitative assessment of multiple antibody targets simultaneously, an advance on previous single-plex ELISA platforms (40). Studies have differed by the timing of serum sampling,

immunoglobulin sub-type targets (e.g. IgM, IgG and/or IgG sub-sets) and how the result was analysed and reported. There were also differences in the reagents and protocols used for the assays.

Table 2 summarises the timing of serum sampling from participants relative to KLH immunisation for the fourteen relevant studies from Table 1. All studies tested KLH antibodies at baseline, then at variable time-points after immunisation, with sampling at Weeks 2 (71%), 4 (43%) and 3 (36%), respectively, the next most frequent. KLH IgG (or an IgG sub-set) was assayed in 14 (100%), IgM in 10 (71%), IgE in 2 (14%) and IgA in 1 (7%) of the studies that measured antibodies.

[Insert Table 2]

Studies have also differed in how the KLH antibody titre was read and presented. For example, standard curves have been generated using sera with known concentrations of KLH antibody, and against these the concentration of anti-KLH antibodies in subjects' samples were interpolated (33,35,38). Other studies have compared the optical density (measure of colour change/light absorbance in wells) of sample sera at defined dilutions with the positive and negative control sera on the same plate which also allowed adjustment for inter-plate variation in absorbance readings (28,31,32). One study assayed KLH IgG and IgM at three dilutions (not specified) and calculated the average across dilutions for analyses (30).

Quantifying the cell-mediated immunity response

Many of the studies reviewed assessed aspects of cell-mediated immunity *ex vivo* following KLH immunisation (28,33,35,37,38,40,42,43,45). The majority of these studies used conventional lymphocyte proliferation assays, with the main difference between them being the incubation

periods of the peripheral blood mononuclear cells (PBMCs) with KLH, ranging from 5 days (28) to 7 days (33).

Cytokine production by stimulated PBMCs following KLH immunisation was assessed in two recent studies. Interferon-γ (IFN- γ) production was determined by ELISPOT assay after thawed PBMCs were incubated with KLH for 20 hours (33). Spazierer et al (38) incubated cells with KLH for 40 hours then tested the supernatant for IL-4, IL-5, IL-10, IL-13 and IFN-γ using antibody-coated magnetic bead assays.

Ferbas et al (40) developed a B-lymphocyte ELISPOT that could enumerate antigen-specific B cells secreting KLH IgG at various time-points post-immunisation, thereby showing the kinetics of the cellular response and the relationship with serum KLH IgG levels. In an innovative study, Kantele et al (43) sorted peripheral blood lymphocyte cell populations by their tissue-specific homing receptors (e.g. L-selectin for lymph node tissue; $\alpha_4\beta_7$ for intestine), then utilised ELISPOT assays to identify which of these cells secreted KLH-specific antibody. This research showed that the immune response following KLH immunisation was characterised by a non-intestinal, systemic homing profile.

Delayed-type hypersensitivity (DTH) testing is a validated *in vivo* test of antigen-specific cell-mediated immunity. A number of the reviewed clinical studies used DTH tests to assess *in vivo* KLH-specific cell-mediated immunity (28,31,32,37,42,44,45). The studies have varied in the initial immunisation KLH dose, as well as formulation and subsequent skin test dose (see Table 1).

There are conflicting data regarding the minimum sensitising and subsequent skin test dose required to induce a reliable DTH response. Grant et al (32) were unable to elicit a DTH response after administration of a 5µg intra-dermal skin test dose three weeks following immunisation with 125µg HMW KLH administered intra-muscularly. Contrasting with this finding, a very early study found that the DTH response was independent of initial sensitising immunisation dose (i.e. 5000µg / 100µg /

1µg were equivalent) (21). However, higher subsequent skin test dose yielded a higher proportion and magnitude of positive DTH responses in a dose dependent manner (i.e. 100µg > 10µg > 1µg) (21). Skin testing with simultaneous doses of 0.1, 1 and 10µg KLH at day 7 and day 14 post-immunisation (with 200µg HMW KLH) achieved a DTH response rate of 68% amongst healthy control participants (although the results at each skin test dose were not provided) (13).

Conclusions

KLH is a potent immunostimulatory antigen that has been used in a number of human clinical research settings and has an excellent safety profile. A robust immune response can be attained from immunisation with a single dose of KLH by various routes and in various doses. Sub-unit KLH needs to be combined with an adjuvant to match the immunogenicity of HMW KLH. There is currently no uniform sampling time/s or laboratory platform for measuring the humoral or cellular KLH-specific response following immunisation. Development of a standardised approach to KLH administration and measurement of antigen specific immune outcomes is required to increase the utility of this very promising agent in human immunotoxicology studies.

Competing interest statement

"All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: AS had scholarship support from the Australian National Health and Medical Research Council for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work".

Acknowledgements

Author contributions:

AS – literature review, drafting and formatting of review

RL, KD and TM – drafting and formatting of review

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Table 1: Clinical studies using keyhole limpet haemocyanin (KLH) administered via a trans-cutaneous route to assess the influence of an exposure or agent on immune response in humans (Studies conducted between 1994 – 2013)

Study and aim	**KLH formulation (Source), dose, route & site of administration**	**Antibody assessment: assay type, target immunoglobulin(s), sampling period relative to immunisation**	**Cell-mediated immunity (*Ex vivo* and *in vivo* testing)**
Ferbas et al (2013)(40) To assess performance characteristics of immunoassays measuring antigen specific response to KLH immunisation in healthy controls and patients with systemic lupus erythematosus	HMW-KLH (ImmuneActivator™, Intracel, Rockville, USA) 1000µg ID Forearm 2 doses: Day 1 & 29	Flow cytometric bead array KLH IgM and IgG; IgG1-4 Baseline and days 7, 14, 28, 35 and 42 post-immunisation	***Ex vivo*:** ELISPOT assay to detect numbers of B cells secreting KLH IgG ***In vivo*:** Not evaluated
Gallegos et al (2013)(30) To examine the effects of mindfulness-based stress reduction on immunological outcomes in older adults	KLH formulation not noted 8, 40, 100, 200, 1000µg KLH IM Deltoid muscle	ELISA (type not-specified) KLH IgM and IgG Baseline, 3 & 24 weeks post immunisation	**Not evaluated**
Boulton et al (2012)(42) To examine the influence of fingolimod therapy on immune responses to immunisation with neo-antigens and recall antigens	Sub-unit KLH (Immucothel®, Biosyn, Carlsbad, USA) 0.5mL(100µg) adsorbed to alum IM Site not specified	Indirect ELISA KLH IgM, IgG Baseline and 1, 2, 3, 5 and 7 weeks post-immunisation	***Ex vivo*:** Not evaluated ***In vivo*:** 10µg KLH ID X-weeks post-immunisation; Anterior aspect of upper or lower arm; Read at 48 hours

Kantele et al (2011)(43) To examine the tissue homing response of lymphocytes following KLH immunisation and secretion of KLH antibodies at a cellular level	HMW-KLH (Pacific Biomarine, Venice, USA) 100 µg SC Site not specified	Quantification of serum KLH antibodies not performed	***Ex vivo*:** ELISPOT assay to detect number of B cells secreting KLH IgG, IgA and IgM after immune-magnetic sorting of lymphocytes by tissue-specific homing receptors ***In vivo*:** Not evaluated
Bingham et al (2010)(36) To examine immunisation responses in patients with rheumatoid arthritis treated with rituximab	HMW-KLH (Intracel, Frederick, USA) Dose not specified SC Site not specified	ELISA (type not-specified) KLH IgG Baseline and 4 weeks post-immunisation	**Not evaluated**
Spazierer et al (2009) (38) To establish an immunisation protocol to induce *de novo* Th2 responses using immunisation with KLH	Sub-unit KLH (Immucothel®, Biosyn Arzneimittel GmbH, Fellbach, Germany) 100µg KLH with alum (dose not specified) IM Site not specified; 3 doses: Day 1, 15 & 29	Indirect and sandwich ELISA KLH IgG1, IgG4, IgE, IgM Baseline and days 14, 28, 42 & 56 post-immunisation	***Ex vivo***: Lymphocyte proliferation assays ***In vivo***: Not evaluated

Grant et al (2008) (32) To examine the effect of aerobic exercise in sedentary older adults on primary immune response to KLH immunisation	HMW-KLH (BCI-ImmuneActivator™, Intracel, Rockville, USA) 125µg IM Deltoid muscle	Indirect ELISA KLH IgG1, IgG2, IgM Baseline and 2, 3 and 6 weeks post-immunisation	***Ex vivo*:** Not evaluated ***In vivo*:** 5µg KLH ID 3-weeks post-immunisation; Timing of reading not specified; Site not specified
Miller et al (2005) (33) To compare the responses to KLH immunisation in healthy adults with those in immunosuppressed patients (cancer & bone marrow transplant recipients)	HMW KLH HMW KLH (Intracel, Rockville, USA) Sub-unit (Biosyn Corp, Carlsbad, USA) 1. Sub-unit KLH 1000 µg 2. HMW-KLH 1000µg 3. Sub-unit KLH 1000µg with Montanide-ISA-51 adjuvant (0.6mL) SC Site not specified	Indirect & sandwich ELISA KLH IgG1, IgG2, IgM Baseline and 4 weeks post-immunisation	***Ex vivo*:** Lymphocyte proliferation assays; ELISPOT assay for cellular responses to KLH ***In vivo*:** Not evaluated
Smith A et al (2004) (28) To examine the effect of distress on primary KLH immunisation response in young adults	Sub-unit KLH (Pierce, Rockford, USA) 100µg KLH adsorbed to 0.9mg alum IM Deltoid muscle	Indirect ELISA; KLH IgG Baseline and 3 weeks post-immunisation	***Ex vivo***: Lymphocyte proliferation assays ***In vivo***: 1 µg ID 3 weeks post-immunisation; Volar aspect of arm; Read at 48 hours

Smith A et al (2004) (29) To examine the effect of psychological distress on DTH response following primary KLH immunisation in young adults	Sub-unit KLH (Pierce, Rockford, USA) 100µg KLH adsorbed to 0.9mg alum IM Deltoid muscle	Not evaluated	***Ex vivo***: Not evaluated ***In vivo***: 1 µg ID 3 weeks post-immunisation; Volar aspect of arm; Read at 48 hours
Smith TP et al (2004) (31) To examine the effect of age and physical activity on primary immune response to KLH immunisation	Sub-unit KLH (Pierce, Rockford, USA) 100µg KLH adsorbed to 0.9mg alum IM Deltoid muscle	Indirect ELISA KLH IgG, IgG1, IgG2 & IgM; Baseline and 1, 2, 3 & 4 weeks post-immunisation	***Ex vivo***: Not evaluated ***In vivo***: 1 µg ID 21 days post-immunisation; Volar aspect of arm; Read at 24, 48, 72, 96 & 120 hours
Boelens PG et al (2004) (44) To examine the effect of severe trauma on early primary immune response to KLH immunisation in relation to low plasma glutathione	KLH formulation/source not stated 500 µg SC Deltoid region	Indirect ELISA KLH IgM, IgA, IgG, IgG1-4 Baseline and days 8 and 13 post-immunisation	***Ex vivo:*** Lymphocyte proliferation assay; Frequency of interferon-γ (Th1) and IL-4 (Th2)-producing T-lymphocytes cells by flow cytometry assays ***In vivo:*** 100 µg ID 14 days post-immunisation; Right gluteal region of leg; Read at 48 hours
Rentenaar RJ et al (2002) (45) To examine the cellular and humoral responses to immunisation in renal transplant recipients receiving different immunosuppressive regimes	HMW KLH (Source not stated) 1000 µg SC Right arm	Indirect ELISA KLH IgG Baseline and day 14 post-immunisation	***Ex vivo:*** Lymphocyte proliferation assay; ***In vivo:*** 1 & 10 µg ID 14 days post-immunisation; Lower arm; Read at 24 hours

Van der Kolk et al (2002) (34) To assess the influence of rituximab on the humoral immune response to immunisation with primary and recall antigens in patients with low grade lymphoma	HMW KLH (Calbiochem, San Diego, USA) 1000 µg SC Site not specified	Indirect ELISA KLH IgG Baseline and 14 days post-immunisation	Not evaluated
Valdez H et al (2000) (37) To assess response to immunisation after prolonged anti-retroviral therapy in patients with HIV	HMW KLH (ImmuneActivator™, PerImmune, Rockville, USA) 1000 µg ID Site not stated	Indirect ELISA KLH IgG Baseline and 2, 6, 12 and 18 weeks post-immunisation	***Ex vivo:*** Lymphocyte proliferation assays ***In vivo:*** ID (dose not specified); 6 and 18 weeks post-immunisation; Read at 48 – 72 hours
Kondratenko et al (1997) (35) To evaluate responses to primary KLH immunisation in patients with immunodeficiency states	HMW KLH (Calbiochem, San Diego, USA) 200 µg ID Upper arm	Indirect ELISA KLH IgG, IgM Baseline and 2 & 4 weeks post-immunisation	***Ex vivo:*** Lymphocyte proliferation assay ***In vivo***: Not evaluated

Legend: IM: Intra-muscular; SC: sub-cutaneous; ID: Intra-dermal HMW: High molecular weight DTH: Delayed type hypersensitivity ELISPOT: Enzyme-linked immunosorbent spot assay;

Table 2: Timing of serum sampling for KLH antibody assays relative to KLH immunisation

Timing of Sample (weeks post-immunisation)	**0 (baseline)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**12**	**18**	**24**
Number of studies (% of studies from Table 1*)	14 (100%)	4 (29%)	10 (71%)	5 (36%)	6 (43%)	2 (14%)	4 (29%)	1 (7%)	1 (7%)	1 (7%)	1 (7%)	1 (7%)

* Fourteen studies from Table 1 were included here that had measured KLH specific antibodies as part of their respective study protocols