Heterogeneous Alterations in Human Alloimmunity Associated with Immunization

Meagan Roddy,[1] Michael Clemente,[1] Emilio D. Poggio,[1] Ronald Bukowski,[2] Snehal Thakkar,[2] Gunter Waxenecker,[3] Hans Loibner,[3] Gottfried Himmler,[3] Donald E. Hricik,[4] and Peter S. Heeger[1,5,6]

Background. The presence of alloantibodies and/or alloreactive T cells in a patient prior to a transplant can impact graft outcome. Environmental factors, including therapeutic vaccinations, may influence the strength and/or specificity of alloimmunity.

Methods. To address this issue, we prospectively evaluated the effects of two different immunization protocols in human subjects on cellular alloimmunity using an IFNγ ELISPOT assay and on alloantibody reactivity by flow cytometric analysis of HLA-coated beads.

Results. Vaccination/immunization was associated with augmentation of cellular and/or humoral alloimmune reactivity in >50% of the test subjects. The effects were heterogeneous in that some detected increases were transient, peaking 30-60 days postimmunization, whereas others persisted for the length of the study. Antibodies reactive to the immunizing agent did not cross react with the detected alloantibodies, suggesting that the augmentation of alloimmune reactivity was most likely due to a nonspecific adjuvant effect from the vaccine.

Conclusions. Therapeutic vaccinations can alter the strength of cellular and humoral alloimmunity in humans. The results suggest that serial immune monitoring of alloreactivity might be beneficial when immunizations are administered to potential transplant recipients.

Keywords: T lymphocyte, Cytokine, Alloreactivity, Immunization.

Alloreactive T cells and alloantibodies are known mediators of acute and chronic allograft injury, processes that limit the lifespan of transplanted organs (1–4). The presence of pretransplant anti-HLA antibodies in a potential recipient, as detected by a positive panel of reactive antibody (PRA) test and/or a positive crossmatch test, are known to increase the risk for early posttransplant injury/graft loss (1,5); transplantation is generally not performed across a positive crossmatch. As the sensitivity of alloantibody testing has improved through the use of flow cytometric bead techniques, it has also become clear that low titers of antidonor HLA antibody not detected in standard crossmatch studies (that do not preclude transplantation) may also predict a poor posttransplant prognosis (6–9).

Studies from our laboratory have shown that antidonor effector/memory T cells can be detected in the peripheral blood of transplant recipients prior to and following transplantation (10–13). The emerging data suggest that the frequency of pre- and posttransplant antidonor T cell immunity positively correlates with poor posttransplant outcome, potentially because such effector/memory T cells are resistant to standard immunosuppression regimens (10,14).

The specificity and or strength of alloimmune reactivity may vary over time, particularly in response to environmental stimuli. PRA values, for example, can change significantly while patients await transplantation (15–17). Because the strength of the alloimmune repertoire prior to transplantation impacts on posttransplant outcome, understanding exogenous factors that influence antidonor immunity prior to transplantation has important clinical implications.

Organ transplantation requires recipient immunosuppression and thereby carries inherent infectious risks. Physicians commonly administer vaccinations against infectious pathogens (i.e. influenza, hepatitis B) in these patients to induce/augment protective immunity. Although such a practice may be efficacious, it is possible that the immunization procedure, as a "nonspecific" proinflammatory stimulus and/or via priming of cross-reactive T or B cells, could en-

hance the strength of any preexisting alloreactivity. Such a theoretical possibility has led some transplant physicians to avoid immunizations proximate to the transplant procedure, but whether immunizations affect alloimmune responses in humans is an issue that has not been carefully studied.

To assess the effects of therapeutic immunizations on human alloreactive immunity we prospectively studied the kinetics and dynamics of cellular and humoral responses to alloantigens in two cohorts of subjects undergoing two separate immunization protocols. The results reveal that such immunization protocols have heterogeneous effects on cellular and humoral alloimmune reactivity, raising important questions about when immunizations should be administered with respect to a potential transplantation procedure and underscoring the potential importance of serial immune monitoring to detect such changes in transplant candidates.

MATERIALS AND METHODS

We studied patients with late-stage malignancies unresponsive to standard therapies and entered into a phase I clinical trial in which they received an anti-idiotypic murine antibody, IGN301, designed to mimic the target tumor antigen Lewis Y. Ten patients had a primary adenocarcinoma of the gastrointestinal tract, five had primary renal cell carcinoma and two had primary small cell carcinoma of the lung. There were 13 men and 4 women, 16/17 patients were Caucasian (1 African American) and the median age of the cohort was 60 years old (range 32-77). After performing screening, imaging studies and baseline laboratory tests, each patient was vaccinated with IGN301 adsorbed on alum, s.c. on days 1, 15, 29, and 57. We obtained peripheral blood samples in heparinized tubes to test for cellular and humoral alloimmunity on day 1 (baseline-prior to the initial immunization), on day 57 and on day 92 after the initial immunization. Samples of peripheral blood lymphocytes (PBLs) from each patient at each time point were assessed for the percentage of T cells by flow cytometry using standard techniques as performed by our laboratory (*12,13*). In all, 32 patients were originally enrolled, but only 17 completed the 92-day study and were used in this analysis.

In a second analysis, we studied peripheral alloimmune reactivity in 15 normal, healthy students attending a local graduate school program during the course of hepatitis B vaccination. Baseline samples were obtained prior to the initial immunization, on day 30 (at the time of a second immunization) and on day 60 or day 180 following the initial immunization. All samples were drawn when patients or volunteers were clinically healthy. None of the volunteers were pregnant or taking medications. Three unimmunized normal healthy patients were serially evaluated concomitantly with this cohort as controls.

All studies in both immunization protocols were conducted after obtaining informed consent and under the approval and guidance of the Cleveland Clinic Foundation's Institutional Review Board.

Isolation of Peripheral Blood Lymphocytes

Patient's PBLs were isolated from heparinized blood samples using Ficoll density-gradient centrifugation (*10,11*). Live cell counts were determined by ethidium-bromide/acri-dine orange staining and visualization under an immunofluorescence microscope. Plasma samples were isolated, aliquoted and frozen at $-80°C$.

ELISPOT Assays for Alloreactive Cellular Immunity

Responder PBLs from each individual were tested in interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assays against a panel of two or three randomly chosen T-cell depleted allogeneic stimulator cells. Each responder was tested against the same set of stimulator cells for each of the time points.

IFN-γ ELISPOT assays were performed as previously described in detail (*10,11*). A total of 300,000 responder PBLs, in 100 μL of T cell medium (93% RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) plus 5% human serum with L-glutamine plus penicillin/streptomycin (BioWhittaker, Walkersville, MD), were immediately placed in a 96-well ELISPOT plate (Millipore, Bedford MA) precoated with capture anti-IFN-γ antibody (Endogen, Woburn, MA). PBLs were stimulated with medium alone (negative control), T cell depleted stimulator cells, mumps antigen (BioWhittaker), and a positive control, phytohemagglutinin (PHA at 1 μg/ml of medium (Murex Diagnostics, Dartford, UK)). Plates were then incubated overnight at 37°C. Following washes with PBS and PBS-Tween, a biotinylated, anti-IFN-γ antibody (Endogen) was added to detect bound cytokine, and the plates were incubated overnight at 4°C. After an additional wash, streptavidin horseradish peroxidase conjugate (Dako, Denmark) was added for 1 h at room temperature. After a final wash, the plates were developed with aminoethylcarbazole (10 mg/ml in N,N-dimethylformamide; Pierce Chemicals, Rockford, IL), prepared in 0.1 M sodium acetate buffer (pH 5.0) mixed with H_2O_2.

The resulting spots were counted with a Series 1 Immunospot computer-assisted ELISPOT image analyzer (Cellular Technology, Cleveland, OH). Results were depicted as the mean number of IFN-γ spots per 300,000 recipient PBLs based on duplicate or triplicate measurements in a given assay. Previous work has demonstrated that <10 spots per 300,000 cells represent background reactivity. A positive test was defined as a >100% increase over baseline and >20 spots per 300,000 PBLs. The well-to-well and assay-to-assay variability is 20-30% (*11*). Negative control wells assessing cytokine production by stimulators alone were included in all assays (< 20 spots per 300,000) and detected spots in these control wells were subtracted from the total number of spots in wells in which responders and stimulators were mixed.

Alloantibody Assessment

Anti-HLA antibody in plasma samples was determined by flow cytometry using HLA class I and class II antigen-coated beads (*13*) as directed by the manufacturer (FlowPRA Screening Test, One Lambda, Canoga Park, CA). All plasma samples from the same patient were tested at the same time along with positive and negative control sera, supplied by the manufacturer. Selected plasma samples that bound to HLA-coated beads were tested for nonspecific binding to control (uncoated) beads, and no binding was detected, confirming that the detected alloantibodies were HLA-specific. Specificities of the anti-HLA alloantibodies were not determined.

Seroconversion and Immune Affinity Chromatography of IGN301 Sera

In the IGN301 trial, seroconversion (as defined by detection of anti-IGN301 antibody in the patient's serum) was assessed by ELISA for anti-IGN301. A positive seroconverter was defined as a patient who had a titer greater than 1:100 for at least two timepoints after start of immunization. In order to purify the overall immune response to the vaccine antigen immune affinity chromatography was used. Patient serum (500 μl) was diluted with affinity chromatography loading buffer (PBS and 0.2 M NaCl) at a 1:10 dilution and loaded onto a Sepharose column conjugated to IGN301. Unbound sample was washed out with loading buffer and elution of bound immunoglobulin was achieved with glycine buffer + 0.2 M NaCl (elution buffer 1) at pH=2.9. Fractions of interest were collected by automated fractionation and immediately neutralized by adding 1 M NaHCO3. Afterwards 200 μl of the fraction pool were mixed with PEG2000 solution (end concentration 0.04% PEG) and IgG and IgM were quantified by SEC-HPLC using a Pentaglobin calibration curve.

ELISA

Immulon flat bottom 96-well ELISA plates (Thermo Electron, San Jose CA) were coated with IGN301 at 1:1000 dilution in PBS, ovalbumin (Sigma, St. Louis, MO, 1 mg/ml) or were not coated with a primary antigen. Eluates from the IGN301-conjugated Sepharose columns (undiluted or at a 1:10, 1:100 or 1:300 dilution) were added to the wells overnight, and blocked with PBS + 1% BSA for 2 h. After three washes with PBS, detecting alkaline phosphatase conjugated antihuman IgG (1:1,000, clone GG-5, Sigma) was added and left overnight at 4°C. The plate was washed three times with PBS followed by a wash in 12.08 g Tris, 17.52 g NaCl, and 0.4 g NaN3 in 1 L H20. Color was developed using PNPP, p-Nitrophenol phosphate, disodium salt, hexahydrate (Research Organics, Cleveland, OH; 50 mg diluted in 30 ml of PNPP buffer: 8.4g NaHCO3, 0.1g NaN3, and 674 μl 4.9 M MgCl2 in 1 L H2O). The plates were read at OD 405.

RESULTS

We initially studied alloimmune reactivity in a cohort of patients with late stage adenocarcinomas undergoing treatment with an experimental murine antibody designed to mimic the target tumor antigen Lewis Y (anti-idiotypic antibody IGN301). Seventeen patients (of an initial 32 enrolled) completed the study. None of these patients was a previous or current transplant recipient. Clinical characteristics of the patients are described in the Materials and Methods.

PBLs from each patient were isolated prior to initiation of the immunization and on days 57 and 92 after the first immunization, and tested against a set of two to three randomly chosen allogeneic stimulator cells in IFNγ ELISPOT assays. PBLs from each patient were recurrently tested against the same set of stimulator cells. Flow cytometric analysis revealed that each PBL sample was comprised of at least 40% T cells (not shown). Representative ELISPOT results from individual patients are shown in Fig. 1A and a summary of the findings is depicted in Fig. 1B. In 6/17 (35%) of the patients we did not detect any change in the frequency of alloreactive PBLs following the immunization. In contrast, an increase in
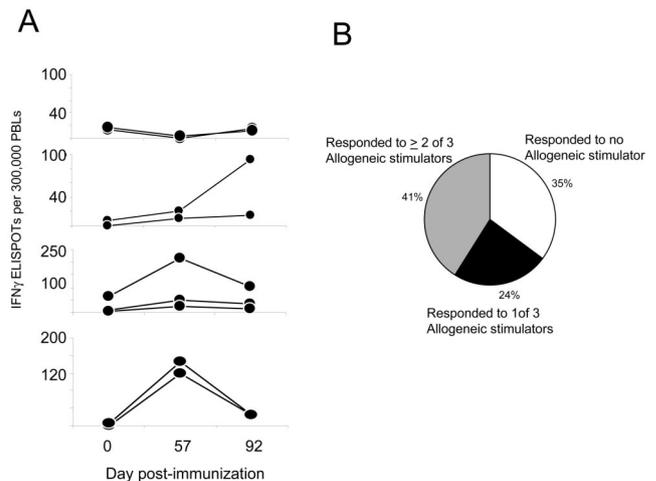

FIGURE 1. Vaccination with IGN301 has heterogeneous effects on cellular alloimmunity. (A) Results from four representative patients are shown individually in each panel. PBLs from each patient were serially tested in IFNγ ELISPOT assays against two or three randomly chosen stimulators and each line represents the response against a different stimulator. Note that the scales on the Y-axes are not identical. (B) Summary of the responses in the entire cohort of 17 patients.

the frequency of alloreactive PBLs (as defined by >20 IFNγ ELISPOTs per 300,000 PBLs and >100% increase over baseline against at least one allogeneic stimulator) was noted in 65% (11/17) of the patients immunized with IGN301. In the majority of cases, the detected increases in alloreactive PBL frequency were transient (higher frequencies on day 57 vs. day 0 with a decline to baseline by day 92) but in several cases the increased reactivity was detectable for at least 92 days (Fig. 1A).

Because of the nature of this phase I clinical trial, no concomitantly studied control patients (unimmunized) were included with this study cohort. We previously showed, however, that alloimmune reactivity in normal volunteers is relatively stable over 6 months with a coefficient of variation of 20-30% (*11*). Thus, >100% increase in detected frequency over baseline strongly suggests that these alterations were related to the immunization procedure, rather than due to interassay variability.

Plasma samples from each patient were also tested for alloantibody reactivity as assessed by FlowPRA bead analysis. Representative results are shown in Fig. 2A and a summary of the findings is shown in Fig. 2B. Although anti-HLA antibodies were not detected at any time in 11 of 17 patients (Fig. 2B, white squares), increases in percent binding to FlowPRA beads were detected in 6 of 17 patients (35%, Fig. 2B, black squares) over the course of the immunization. We did not have access to transfusion or pregnancy histories in these patients, but only two of the six individuals were female. In four of the patients, the detected increased reactivity was directed at both class I and class II alloantigens (one of these was female). One patient developed increased reactivity to only class I HLA and one patient developed reactivity to only class II HLA following the immunization. In contrast to the transient increase noted for the cellular alloimmunity above, the
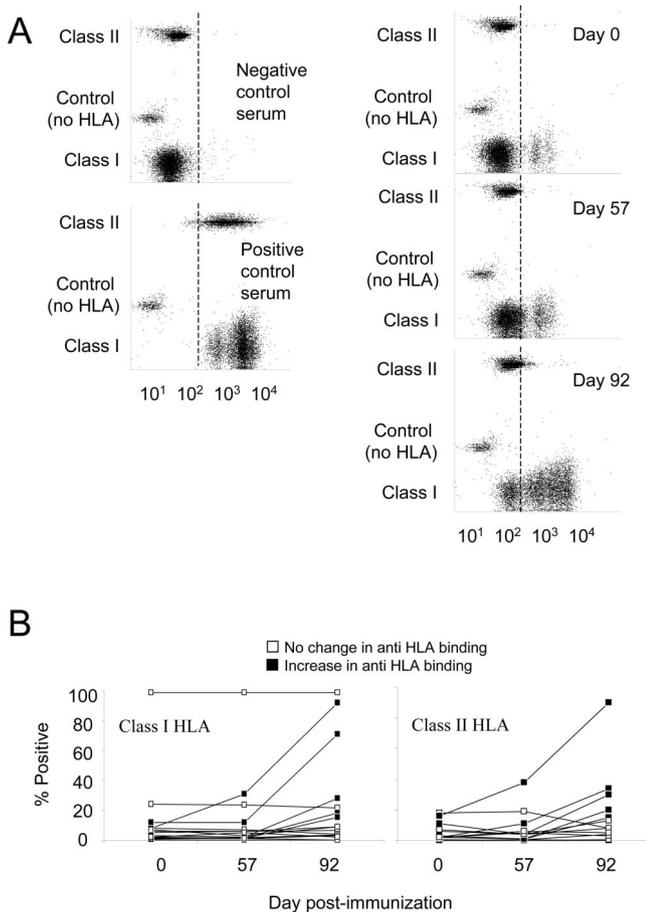
FIGURE 2. Vaccination with IGN301 has heterogeneous effects on humoral alloimmunity. (A) Representative flow cytometry plots depicting increased binding to the class I HLA FlowPRA beads over time (day 0, 57 and 92 shown on right three panels) coincident with the immunization. Dotted line represents the cutoff between positive and negative derived from manufacturer-supplied positive and negative control sera (shown on left two panels). Note that there was no binding to control beads (no HLA) demonstrating the HLA-specificity of the detected positive results. (B) Kinetics of quantified FlowPRA results for all patients with either an increase in binding to class I (left) or class II (right) HLA molecules. In those patients with augmented binding to FlowPRA beads associated with the immunization (n=6), four developed enhanced anticlass I and anticlass II alloantibody binding, one developed enhanced anticlass I antibody alone and one developed enhanced anticlass II antibody alone.

increases in anti-HLA reactivity were most notable towards the end of the study period (at least through day 92 postimmunization). Also notable was that an increase in FlowPRA reactivity was found in patients who had low but detectable (< 5%) baseline positive reactivity. This latter result raised the possibility that the immunization led to nonspecific enhancement of preexisting memory alloimmunity, a hypothesis tested further below.

All of the immunized patients seroconverted as assessed by detection of anti-IGN301 antibody in the serum (not shown). We next asked whether the induced serum anti-

IGN301 antibodies cross-reacted with allogeneic HLA molecules. Anti-IGN301 was isolated from the serum of four patients with detected increases in anti-HLA alloantibodies following immunization using a column containing IGN301-coated Sepharose beads. The eluates from each of these patients contained human immunoglobulin that reacted with IGN301 but not a control protein, ovalbumin (Fig. 3A, B). However, the eluates did not react to HLA molecules by Flow-PRA analysis (Fig. 3C). This was true despite the fact that all of the original plasma samples from these patients reacted to the FlowPRA beads.

The changes in alloreactivity associated with this immunization are summarized in Fig. 4. Seven patients (41%) developed enhanced cellular alloimmunity without altering humoral alloimmunity, four patients (24%) developed enhanced humoral alloreactivity without a detected effect on cellular alloreactivity, and four patients (24%) developed changes in both cellular and humoral alloreactivity. No detectable change in either cellular or humoral alloreactivity was noted in 2 of the 17 (12%) patients.

In a second cohort of subjects, we prospectively assessed cellular and humoral alloreactivity during a hepatitis B immunization. Fourteen nonimmunized healthy volunteers (all < 30 years old), taking no medications, were administered the hepatitis B vaccine as part of routine medical care. Immunizations were administered on day 0 and day 30. PBLs were obtained on day 0, 30 and either 60 or 180, and were tested in IFNγ ELISPOT assays against a panel of three differ-
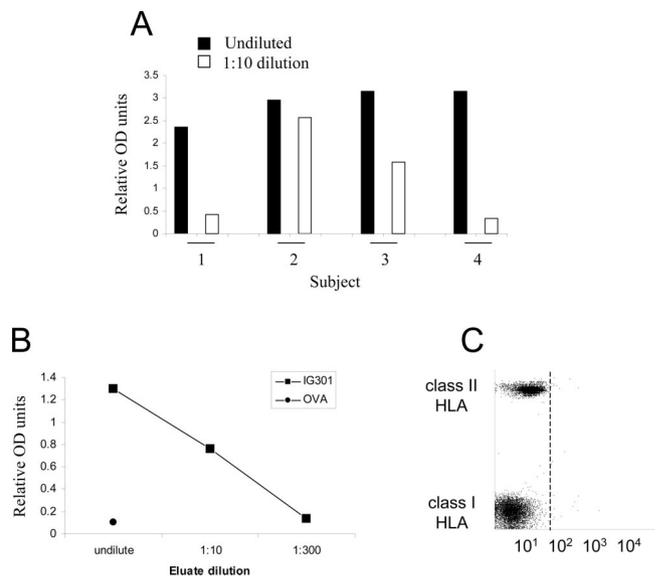

FIGURE 3. Antibodies to IGN301 do not cross react with HLA molecules. Serum samples from four patients with anti-HLA antibodies by FlowPRA were obtained on day 92 and the anti-IGN301 fraction was enriched through binding to IGN301-linked sepharose beads as described in Materials and Methods. The eluates were tested for the presence of total human immunoglobulin (A) and reactivity to IGN301 at varying eluate dilutions (B) by ELISA and for binding to HLA molecules by FlowPRA (C). Panels B-C show results from one patient, panel B includes reactivity to a specificity control (binding to ovalbumin, ova). The results shown are fully representative of the four patients tested.
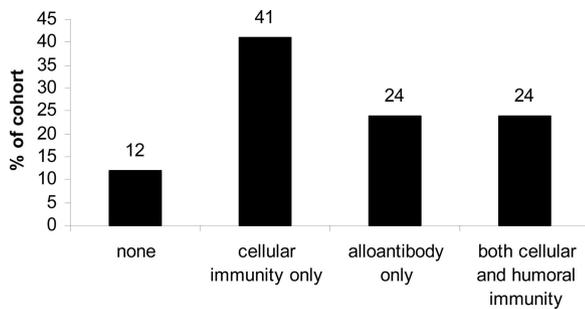
FIGURE 4. Heterogeneity of induced changes in alloimmunity following vaccination with IGN301. The percentage of patients in the cohort (n=17) that had either no change in cellular or humoral alloimmunity, had an increment in either cellular or humoral alloimmunity, or developed an increment in both cellular and humoral alloimmunity is depicted.

ent allostimulators obtained from unrelated normal volunteers. All responders were tested against the same set of stimulators over the course of the study. PBLs from 11/15 (73 %) of the immunized volunteers responded at significantly higher frequency (>20 IFNγ ELISPOTs/300K PBLs and $>100\%$ increase over the baseline value) to at least one allogeneic stimulator following the initial immunization (Fig. 5A, B). The detected increased frequencies of alloreactive PBLs were transiently detected at 30 days postimmunization in the majority of these individuals, but elevated frequencies of alloreactive PBLs were noted on days 60-180 in several situations (Fig. 5A). Notably, PBLs from nonimmunized normal volunteers (Fig. 5C) tested over the same time period responded at similar frequencies to allogeneic stimulators at all three time points, suggesting that the detected increases in the immunized patients were caused by the immunization. Plasma samples obtained at each time point were also tested for alloantibody. In contrast to the cohort of adenocarcinoma patients, all of the students in this younger and healthy cohort had 0% PRA at baseline, and none developed a positive PRA during the study period (not shown).

DISCUSSION

Because the immunosuppressed state posttransplant puts the patient at risk for opportunistic infections and may prevent effective immune responses to vaccinations, pretransplant immunizations may be most efficacious at inducing protective immunity. On the other hand, nonspecific immune activation ("adjuvant effect") and/or induction of cross reactive immunity coincident with vaccinations may lead to enhanced antidonor alloresponses, a consequence that could adversely affect graft outcome. Such theoretical considerations along with anecdotal reports of acute rejection episodes closely following immunizations, have led many physicians to avoid therapeutic immunizations in the peritransplant period (when the risk of acute rejection is highest). Most studies of immunization in transplant recipients have focused on efficacy of the vaccine (18–23)) and there has been little published data evaluating whether and how immunizations affect alloimmunity in humans. The findings in this report show, in two different patient populations, that potentially protective vaccinations can have heter-
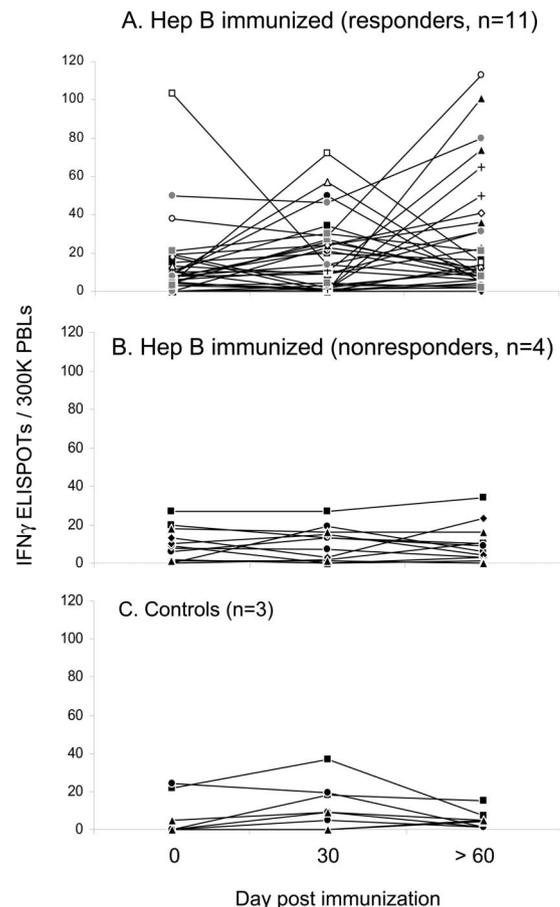

FIGURE 5. Hepatitis B vaccine has heterogeneous effects on cellular alloimmunity in healthy volunteers. (A) Kinetics of quantified ELISPOT results in 11 immunized subjects whose PBLs exhibited an increase in frequency of IFNγ-producing alloreactive PBLs reactive to at least one of three different stimulator cell populations. Each symbol represents the response from a single patient and there are three lines for each symbol depicting the responses against each of the individual stimulator cells tested. PBLs from five patients exhibited enhanced reactivity ($>20/300,000$ PBLs and $>100\%$ increase over baseline) to 1/3 allogeneic stimulator cells coincident with the immunization, PBLs from five patients exhibited enhanced reactivity to 2/3 stimulators, and PBLs from one patient exhibited enhanced reactivity to all three stimulator cells. (B) Kinetics of quantified ELISPOT results in four immunized subjects whose PBLs did not respond to any of the stimulators at any time point tested (not shown). Each symbol represents the response from a single patient and there are three lines for each symbol depicting the responses against each of the individual stimulator cells tested. C. Kinetics of quantified ELISPOT results in nonimmunized control patients studied over the same time period. Each symbol represents the response from a single patient and there are two to three lines for each symbol depicting the responses against each of the individual stimulator cells tested.

ogenous and nonpredictable effects on human T and B cell alloimmune reactivity.

Augmented T cell alloimmunity coincident with immunization was detected in a significant proportion, but not

all of both study populations. In the majority of those who developed alterations in alloreactivity, the augmentations were transient, but in several cases the changes were detectable for the entire study period. Similarly, increases in alloantibodies were detected in a significant proportion (but certainly not all) of our study patients, and were often detected for the length of the study period (90-180 d). The specificities of the induced alloantibodies in these six individuals were not determined so we cannot address whether any particular HLA allele is preferentially associated with the risk of developing alloantibodies under these conditions.

The immunization-associated alterations are likely to be due to augmentation of preexisting memory T and B cells boosted by the adjuvant/proinflammatory environment, rather than due to priming of antigen-specific immunity with cross reactivity to alloantigens. The augmented immunity occurred only in patients with low but detectable alloimmunity at baseline and the induced responses were reactive to many alloantigens. Moreover, specific characterization of the antibody fraction in IGN301-immunized patients showed no evidence of cross-reactive immunity. Although we do not have specific information regarding previous exposure to alloantigens through pregnancy and blood transfusion (none were previous transplant recipients), it is noteworthy that the immunization-induced increases in alloantibody were detectable only in the older patient cohort with malignancies. Older patients may be more likely to have larger pools of memory T and B cells than younger patients and malignancies may dysregulate immune function, providing potential explanations to account for this observation.

To our knowledge, there were no intercurrent illnesses or untoward events that would have influenced our results, but we cannot be certain that all detected changes in the frequency or strength of the alloimmune reactivity was due to the original immunization. Moreover, our study was not designed to comprehensibly evaluate the kinetics of the detected immune reactivity, and it is possible that the strength of the induced alloimmune responses would wax or wane further with additional time.

In summary, our results show that prophylactic immunizations can influence alloimmune reactivity, a finding with potentially important clinical implications. Although we have not yet studied the effects of vaccinations in transplant recipients, it is tempting to speculate that immunization-induced alterations in alloantibody titers or alloreactive T cell immunity may underlie the anecdotal associations between administration of vaccines (e.g., influenza vaccine) and acute rejection episodes. In addition, because pretransplant alloimmunity can affect posttransplant outcome, our data raise the possibility that pretransplant vaccine-induced increases in alloimmunity could negatively impact posttransplant graft function. Serial testing of antidonor immunity using standard methods described herein, particularly before and after administration of vaccines, has the potential to function as a risk assessment approach for predicting incipient graft injury. However, controlled prospective studies need to be performed before we can conclusively state that immune monitoring will be beneficial for guiding changes in immunosuppression and/or providing evidence that the transplant procedure be delayed until the alloimmune reactivity wanes.

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