

Report From the 1st International NOD Mouse T-Cell Workshop and the Follow-Up Mini-Workshop

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A workshop on autoreactive T-cell responses in NOD mice was held to optimize autoreactive T-cell detection methodologies. Using different proliferation assay protocols, 1 of the 11 participating laboratories detected spontaneous T-cell responses to GAD(524-543) and insulin(9-23) in their NOD mice. Two other laboratories were able to detect autoreactive responses when using enzyme-linked immunosorbent assay (ELISPOT) and enzyme-linked immunosorbent assay (ELISA) analysis of cytokines in culture supernatants, suggesting that these assays provided greater sensitivity. To address the divergent findings, a follow-up mini-workshop tested NOD mice from four different colonies side-by-side for T-cell proliferative responses to an expanded panel of autoantigens, using the protocol that had enabled detection of responses in the 1st International NOD Mouse T-Cell Workshop. Under these assay conditions, 16 of 16 NOD mice displayed proliferative responses to whole GAD65, 13 of 16 to GAD(524-543), 9 of 16 to GAD(217-236), 7 of 16 to insulin(9-23), and 5 of 16 to HSP277. Thus, spontaneous proliferative T-cell responses can be consistently detected to some β -cell autoantigens and peptides thereof. Overall, the results suggest that more sensitive assays (e.g., ELISPOT, ELISA analysis of cytokines in supernatants, or tetramer staining) may be preferred for the detection of autoreactive T-cells. *Diabetes* 50:2459-2463, 2001

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Additional information can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent assay; SI, stimulation index; UCLA, University of California, Los Angeles.

The results of the recent Human T-Cell Workshops demonstrated that significant problems exist in documenting T-cell responses to β -cell autoantigens in humans (1). With this in mind, it was thought that by stepping back to the NOD mouse model, it would be possible to optimize protocols for detecting spontaneous autoreactive T-cell responses. The lessons learned could then be applied to the characterization of human autoreactive T-cells. Because the NOD mouse is an inbred strain, it should provide a consistent source of well-defined autoantigen-specific T-cell responses that laboratories throughout the world could use as a standard to assess the sensitivity of their T-cell characterization assays.

However, contrary to the notion that NOD mice are immunologically uniform, different laboratories have reported wide variations in NOD mouse T-cell responses to different β -cell antigens. Whereas some laboratories can detect autoreactive T-cells directly *ex vivo*, other laboratories find that β -cell-reactive T-cells must be pre-expanded, or cloned, to characterize them. Laboratories that can detect proliferative responses to whole β -cell autoantigens directly *ex vivo* often do not detect responses to peptides that were identified as containing autoantigen target determinants in the NOD mice of another colony. It is unclear whether these differences arise from differences in the autoimmune pathology in NOD mice of different colonies or from technical aspects of the T-cell characterization procedures. Therefore, a NOD mouse T-cell workshop could also begin to address the basis for the observed variations in T-cell autoreactivity.

RESULTS AND DISCUSSION

Results from the 1st International NOD Mouse T-Cell Workshop. In organizing the 1st International NOD Mouse T-Cell Workshop (hereafter referred to as the 1st Workshop), it was decided that because of the difficulties in obtaining sufficient amounts of purified whole β -cell autoantigens (such as GAD65, IA-2, and HSP65) for distribution, synthetic peptides containing defined target determinants would be tested. The use of synthetic peptides would also circumvent issues concerning the purity of the antigen. Furthermore, these peptides could be readily synthesized by other researchers.

Three peptides that are immunogenic in NOD mice were distributed: 1) mouse GAD(524-543) (also termed GADp35;

TABLE 1
Detection of T-cell responses

Assay	Result
Laboratory no. 1 Proliferation	Responses to GAD(524-543) and insulin (9-23) detected (SIs ~10)
Laboratory no. 2 Proliferation	No responses detected
Laboratory no. 3 Proliferation	No responses to GAD(524-543) or insulin(9-23) Responses detected to GAD(217-236) (average SI ~3)
ELISA analysis of cytokines in culture supernatants	Responses detected to GAD(524-543) and insulin(9-23)(~5-fold over background) and to GAD(217-236) (~43-fold over background)
Laboratory no. 4 Proliferation	No responses detected
Laboratory no. 5 Proliferation	No responses detected
Preexpansion of cells followed by ELISPOT	Responses detected to GAD(524-543) (>100 spots over background) but not to insulin(9-23)
Laboratory no. 6 Proliferation	No responses detected
Laboratory no. 7 Proliferation	No responses detected
Laboratory no. 8 Proliferation	No responses detected
Laboratory no. 10 Proliferation	No responses detected
ELISPOT	No responses detected
Laboratory no. 12 Proliferation	No responses detected
CFSE-FACS	No responses detected
ELISPOT	No responses detected
Laboratory no. 13 Proliferation	No responses detected

FACS, –fluorescence activated cell sorter.

SRLSKVAPVIKARMMEYGT (2); 2) insulin B chain(9-23) (SHLVEALYLVCGERG) (3); and 3) a control self-peptide from mouse serum albumin, MSA(560-574) (KPKATAEQLKTVMD) (4). All of these peptides were >95% pure and had been functionally tested in the laboratories that had contributed them. Each peptide was dissolved to 700 $\mu\text{mol/l}$, and aliquots were sent frozen in coded tubes to the participating laboratories.

The participating laboratories tested for splenic T-cell proliferative responses to these peptides (and any other antigens they wanted to add) in female NOD mice 7–10 weeks of age as well as age- and sex-matched wildtype mice (e.g., BALB/c and C57BL6). Neither the proliferation protocol nor the media were standardized. Each laboratory used its own preferred assay protocol and reagents. As a reference, we distributed a detailed T-cell proliferation protocol (from laboratory no. 1). Participating investigators were encouraged to perform other assays as well, such as enzyme-linked immunospot assay (ELISPOT) and enzyme-linked immunosorbent assay (ELISA) of cytokines in the media, to cross-compare the sensitivity of the assays.

The following participants' laboratories were included: L.C. (Paris), L.C.H. (Melbourne, Australia), K.H. (Denver, CO), D.L.K. (Los Angeles), N.S. (La Jolla, CA), E.S. (La Jolla), B.S. (London, ON), R.T. (Chapel Hill, NC), D.W. (Denver, CO), M.V.H. (La Jolla), and L.W. (New Haven, CT). Each laboratory was assigned a code number, and the results were presented in an anonymous fashion at the Juvenile Diabetes Foundation International–sponsored

conference “Predicting Diabetes and Response to Preventive Therapy: Can Animal Models Provide Lessons for Man?” at Snow Mountain, Colorado, October 2000.

Altogether, only 3 of the 11 participating laboratories could detect T-cell responses to either insulin(9-23) or GAD(524-543) in their NOD mice (Table 1). There was no apparent association between the ability to detect responses and the origin of the NOD mouse colony. Among the three laboratories that detected responses, one colony was expanded from Jax NOD mice (laboratory no. 3), and two were expanded from Taconic Farms NOD mice (laboratory nos. 1 and 5). Of the >90 control wild-type mice tested among the 11 participating laboratories, only 1 mouse had a response to an autoantigen that was considered to be significant.

Among the three laboratories that did detect responses in their NOD mice, the following results were reported:

- Laboratory no. 1 used a proliferation assay and detected strong responses to both GAD(524-543) and insulin(9-23) in all of the NOD mice they tested, with stimulation indexes (SIs) of ~10.
- Laboratory no. 3 tested spleen cells from the same individual mice for both antigen-specific proliferative responses and induced cytokines in culture supernatants by ELISA. Whereas their NOD mice displayed no proliferative responses to GAD(524-543) or insulin(9-23), most of the mice responded to another GAD peptide, GAD(217-236) (with an average SI of ~3). The

cytokine assays proved to be far more sensitive. Responses to GAD(524-543) and insulin(9-23) could be clearly detected (with mean γ -interferon [pg/ml] \sim 5-fold over background), and the response to GAD(217-236) was very strong (43-fold over background). Their NOD mice displayed very strong responses to whole GAD65 by both types of assays.

- Laboratory no. 5 did not detect any responses by proliferation. However, when the spleen cells were pre-expanded with antigen *in vitro* and then analyzed by ELISPOT, they found strong responses to GAD(524-543) (with >100 spot-forming colonies over background) but no significant responses to insulin(9-23).

These results left open the question of whether fundamental differences exist in the autoimmune responses of NOD mice from different colonies, or whether the divergent findings stemmed from technical differences in the assays. The results also raised further questions as to whether the appropriate peptides were tested, whether whole β -cell autoantigens are necessary to reliably detect autoreactive T-cell responses, and whether proliferation assays are sensitive enough to directly detect spontaneous β -cell autoantigen-specific T-cell responses.

Follow-up mini-workshop. To help resolve these issues, a follow-up mini-workshop was carried out in which NOD mice from different colonies were tested side-by-side for proliferative T-cell responses using the protocol and reagents that had enabled detection of responses in the 1st Workshop (from laboratory no. 1). Because shipping NOD mice long distances can greatly affect their T-cell responses (unpublished observations, D.K.), it was necessary to find several NOD mouse colonies that were in close proximity so that the mice could be brought to a central testing location with minimal stress and immediately tested. Four of the laboratories participating in the 1st Workshop are located in Southern California (N.S., E.S., and M.V.H. [La Jolla] and D.L.K. [Los Angeles]) and agreed to participate in the follow-up mini-workshop.

The panel of antigens to be tested was expanded to include GAD(217-236) (also termed GADp15; EYVTLKKM REIIGWPGGSGD), HSP277 (VLGGGCALLRCIPALDSLTP ANED) (5), and whole GAD65. The batch of HL-1 media that was used had been pretested in other assays. On the day of the assay, NOD mice, or isolated spleen cells, were brought to the Sercarz laboratory and tested simultaneously for proliferative responses. The procedures were overseen by one of the investigators (D.Z.) to provide consistency in the preparation and plating of the cells.

We tested four female NOD mice 7–10 weeks of age from each colony, along with 7- to 9-week-old BALB/c mice as controls. The highest mean proliferative response was plotted as an SI. The responses from individual mice from each of the colonies were plotted (Fig. 1).

We found that NOD mice from two colonies (laboratory nos. 1 and 2) had larger-magnitude responses, and we set an SI of 3.0 as the level of a significant response. Mice from the two other laboratories (laboratory nos. 3 and 4) had lower-magnitude responses, and for these we set an SI of 2.1 as the level of a significant response. The results reveal the following:

- All of the NOD mice (16 of 16) from different colonies exhibited strong spontaneous proliferative T-cell responses to whole GAD65 (with SIs ranging from 4 to 25). We have used a separate, larger SI scale to indicate the responses to GAD65 in most of the graphs (Fig. 1). None of the control BALB/c mice responded to this GAD65 preparation. Because sufficiently pure preparations of GAD65 for T-cell characterization assays have been difficult to produce, the protocol used by the Sarvetnick laboratory to generate the GAD65 has been described in an online appendix at <http://diabetes.diabetesjournals.org>.
- Of the 16 NOD mice tested from the four laboratories, only 1 displayed a small but significant response to the control MSA(560-574) peptide. (Note that we have plotted the highest response at any peptide concentration, and this data point was an outlier.)
- Significant proliferative responses were detected to GAD(524-543) in 3–4 of the 4 NOD mice that were tested from each colony. Thus, spontaneous responses to GAD(524-543) can be consistently detected in NOD mice from different colonies (at the tested age range).
- NOD mice from laboratory nos. 1 and 4 also consistently displayed responses to GAD(217-236) (with 4 of 4 and 3 of 4 mice responding, respectively) and to insulin(9-23) (with 3 of 4 mice responding). However, only one, or none, of the NOD mice from laboratory nos. 2 and 3 responded to these peptides. This could be because of differences in T-cell antigen specificity, precursor pool sizes, determinant spreading patterns, or antigen-presenting cells among NOD mice from different colonies. It is also possible that the differences reflect the origin of the colonies—laboratory nos. 1 and 4 used NOD mice from Taconic Farms (which were expanded from breeders received 6 years ago or used a few weeks after delivery from Taconic Farms, respectively), whereas laboratory nos. 2 and 3 used mice that were expanded from Jax NOD breeder mice (which were received \sim 3 years ago). Finally, the lower proportion of NOD mice from laboratory nos. 2 and 3 responding to GAD(217-236) and insulin(9-23) may merely reflect that these mice were slightly younger (\sim 7 weeks in age versus \sim 10 and 8 weeks in age for the mice from laboratory nos. 1 and 4, respectively), and autoimmunity may not have spread yet to these determinants as fully (6).
- Generally, the responses to GAD peptides were smaller than those to whole GAD65, which was expected because the whole antigen contains many determinants. Responses to peptides may also have been more difficult to detect as a result of determinant spreading, because the response to any particular determinant may be waxing or waning.
- Whereas the control BALB/c mice did not exhibit responses to GAD65, GAD(524-543), GAD(217-236), insulin(9-23), or MSA(560-574), they did respond to HSP277. We have not seen this before in our other studies. This response is unlikely to be caused by immunostimulatory impurities in the peptide, because many NOD mice did not respond to this peptide.

These results demonstrate that proliferation assays can be used to detect responses to both whole autoantigens and

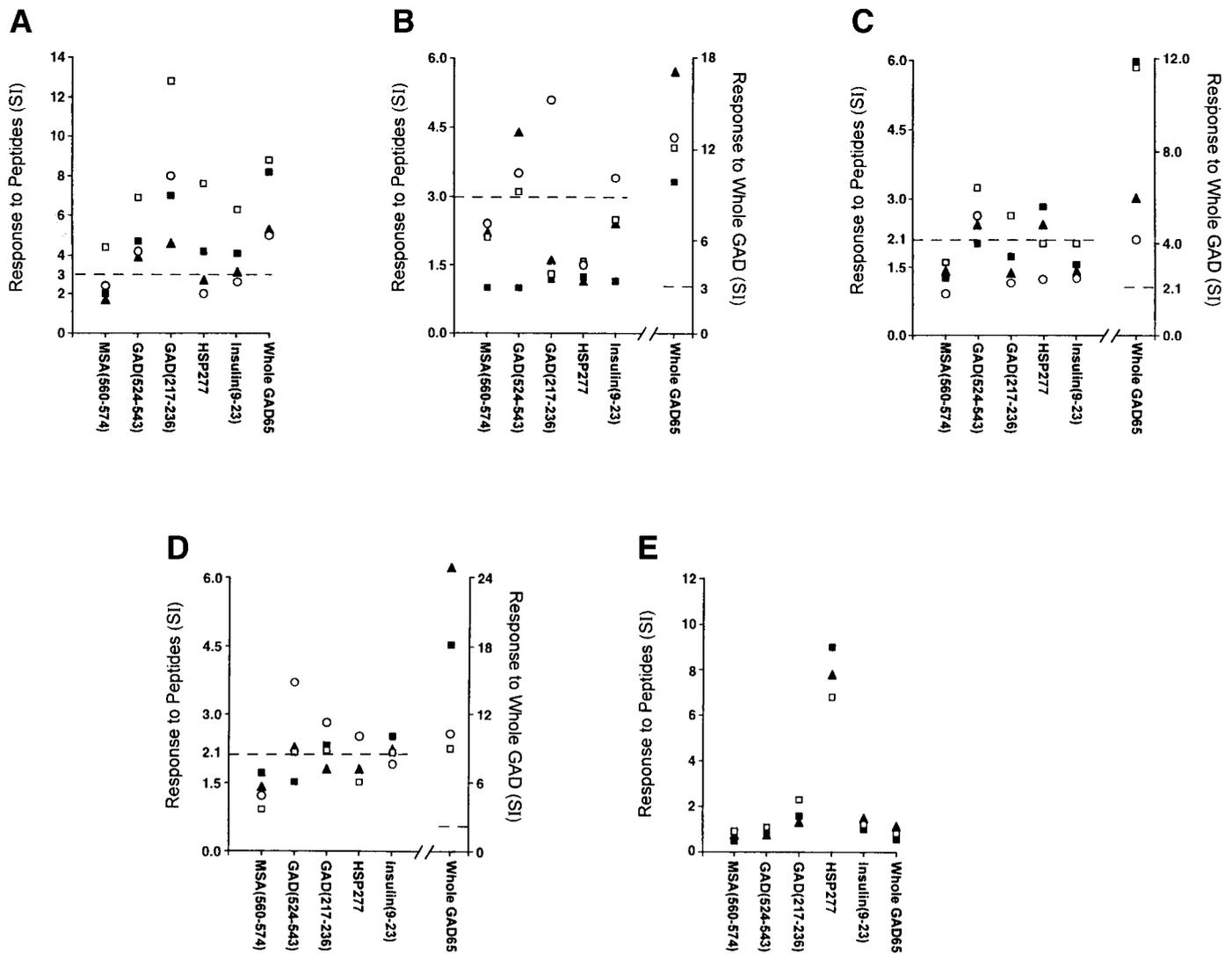


FIG. 1. Proliferative splenic T-cell responses in individual NOD mice from different colonies. Shown are results for NOD mice from laboratories 1 (A), 2 (B), 3 (C), and 4 (D) and for the control BALB/c mice (E). The GAD(524-543), insulin(9-23), and MSA(560-574) peptides were from the same batches distributed for the 1st Workshop. We tested for responses to peptides at 2, 7, and 20 $\mu\text{mol/l}$ and to whole GAD65 at 2, 10, and 20 $\mu\text{g/ml}$. The GAD(524-543), insulin(9-23), HSP277, and MSA(560-574) peptides were added to cultures in a blinded fashion. Four NOD mice from each NOD mouse colony were tested. The highest mean response at any antigen dosage (in triplicate) was plotted as an SI for each mouse. Individual mice are represented with a symbol. Background counts (media alone) ranged from 354 to 1,833 cpm for all of the tested NOD splenic T-cells. Spleen cells from UCLA NOD mice were isolated at UCLA and transported to La Jolla [at ambient temperature] and plated 4 h later alongside cells from the other NOD mouse colonies; this appeared to cause some attrition of their responses.

the peptides thereof. Altogether, all (16 of 16) NOD mice tested responded to whole GAD65, 13 of 16 to GAD(524-543), 9 of 16 to GAD(217-236), 7 of 16 to insulin(9-23), and 5 of 16 to HSP277. However, the magnitude of the responses differed between NOD mouse colonies, and many of the responses were only just above the level considered to be significant. Accordingly, laboratories with NOD mice that have lower-magnitude responses may want to consider using T-cell characterization assays with greater sensitivity. It remains to be determined whether the differences between NOD mouse colony responses arose from variations in their autoantigen recognition or determinant spreading patterns, or merely from differences in the ages of the mice that were tested.

The results also suggest that technical aspects of the proliferation assay are critical to its success. Whereas proliferative T-cell responses were detected in only 1 of the 11 NOD mouse colonies tested in the 1st Workshop,

proliferative responses were detected in all 4 of the NOD colonies tested in the follow-up mini-workshop. The proliferation protocol that was used in the mini-workshop is available at <http://diabetes.diabetesjournals.org>. It should be noted, however, that this is the reference protocol that was distributed for the 1st Workshop, and several laboratories (including laboratories that participated in the mini-workshop) had tried this protocol in the 1st Workshop and did not detect responses. Thus, subtle technical aspects (e.g., in handling splenic cells or in the choice of media) may be crucial to the success of the assay. Notably, it has taken many years to standardize autoantibody detection assays, which are less technically demanding than T-cell proliferation assays.

Finally, several workshop participants felt that proliferation assays were not preferable for detecting autoreactive T-cells, because this assay is not highly sensitive and may be affected by regulatory responses. Indeed, in the 1st

Workshop, two laboratories that could not detect autoreactive T-cell responses by proliferation assays were able to detect them through ELISPOT or ELISA for cytokines. Furthermore, the ELISPOT, intracellular cytokine staining, and tetramer staining assays allow the frequency and avidity of autoantigen-reactive T-cells to be determined. ELISPOT, ELISA for cytokines, and intracellular cytokine staining assays can also provide information on regulatory responses.

The detection of autoreactive T-cell responses in human peripheral blood mononuclear cells is likely to be significantly more challenging than the responses we have tried to detect in splenic cells from NOD mice which have rapidly progressing insulinitis. Accordingly, it will be important to further develop and standardize more sensitive autoreactive T-cell detection and characterization technologies.

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