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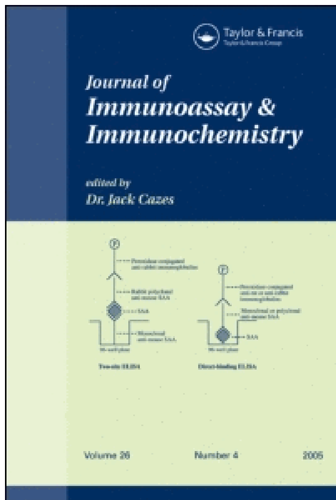

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Modification of T-Cell Antigenic Properties of Tetanus Toxoid by SDS-Page Separation. Implications for T-Cell Blotting

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MODIFICATION OF T-CELL ANTIGENIC PROPERTIES OF TETANUS TOXOID BY SDS-PAGE SEPARATION. IMPLICATIONS FOR T-CELL BLOTTING

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ABSTRACT

Using Tetanus Toxoid (TT) as a model antigen the T-cell Blotting method was evaluated. Peripheral blood mononuclear cell (PBMC) cultures were stimulated by blotted nitrocellulose-bound TT or soluble TT. SDS-Poly-Acrylamide-Gel-Electrophoresis separated TT only induced proliferation in 20% of the PBMC cultures whereas proliferation was induced in 79% of the same cultures offered similar treated TT (except for the PAGE separation). When T-cell blotting was performed with TT separated in a SDS-agarose matrix, proliferation was induced in 80% of donors responding to soluble TT. The results show that SDS-PAGE alters the ability of TT to induce T-cell proliferation, possibly due to unpolymerized acrylamide binding to proteins during SDS-PAGE. The use of SDS-PAGE T-cell blotting in the screening for T-cell antigens must therefore be reconsidered. We suggest the use of SDS-Agarose Gel Electrophoresis as an alternative when doing T-cell blots.

(KEY WORDS:T-cell Blot, Antigen modification, SDS-PAGE, Acrylamide)

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INTRODUCTION

T-cells recognize antigens presented on the surface of antigen presenting cells (APC) in context with MHC-molecules. Protein antigens are taken up by APC and degraded in subcellular compartments in which they combine with the MHC-molecules. Export of the MHC-antigen complex to the surface of the APC makes the antigen accessible for recognition by the T-cell receptor/CD3-complex, as reviewed by Brodski and Guagliardi (1).

The identification of T-cell antigens in complex antigen mixtures has long lacked a simple and reliable approach. As an alternative to traditional biochemical separation methods an approach for direct characterization of approximate molecular weight of T-cell antigens was introduced by Young and Lamb (2). In this method, known as T-cell blotting, protein mixtures are separated by SDS-PAGE and subsequently blotted to a nitrocellulose membrane, which is then used to stimulate T-cells in cultures. PBMC (3-10), T-cell lines and T-cell clones (2,11-15) have been used as responder cells in such assays. The use of T-cell blotting has since been employed to screen for T-cell antigens in protein mixtures derived from Influenza virus (2), *Leishmania* (5,7-9,12,13,16), *Mycobacteria* (3,4,6,11,14,17), *Plasmodium falciparum* (10) and *Chlamydia trachomatis* (15). The value of the T-cell blotting method has been impeded by findings indicating that antigens separated by SDS-PAGE and blotted to nitrocellulose were unable to induce or induced low proliferative responses, even though the crude antigens loaded to the SDS-PAGE gels were excellent T-cell stimulators (4-6,8,17). The lack of response to the blotted antigens, has been explained by incomplete transfer of antigens to the nitrocellulose membrane or lowered immunogenicity of the antigen, due to denaturation or destruction of T-cell epitopes during SDS-PAGE, reviewed by Lamb et al. (18).

In the previous reports it has been impossible to compare the response obtained with soluble crude antigens and responses obtained by nitrocellulose bound antigens, because the soluble antigens used in the T-cell assays did not migrate as one single band during electrophoresis. Therefore, it has not been possible to assess to which degree T-cell

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blotting diminishes the ability of antigens to stimulate T-cells and to identify the steps in the T-cell blotting technique, that are responsible for reducing this ability. In this study we have evaluated these aspects of the T-cell blotting technique by comparing the proliferative responses obtained with a soluble protein antigen and the same antigen bound to nitrocellulose. Tetanus toxoid (TT) was chosen as a model protein because TT migrates as a single band in SDS-PAGE, and TT is known to induce antigen specific T-cell proliferation in vitro. A study was also undertaken to investigate if the observed differences in T-cell stimulatory capacity were due to differences in the tranfer of TT, from the two gel matrices to the nitrocellulose membrane, used in the T-cell blotting experiments.

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MATERIALS AND METHODS

Antigens.

Tetanus toxoid (TT) was obtained from Statens Seruminstitut (Copenhagen, Denmark). The final concentration of TT was 8.8 µg/ml (1.5 µg/culture well) when used as a soluble antigen in or 1.5 µg/nitrocellulose disk when dot blotted onto a nitrocellulose membrane. Concentrations used for T-cell blotting were varied as indicated. In a co-incubation experiment varying concentrations of acrylamide and TT were co-incubated in PBS for four hours at 37°C, and added to T-cell cultures. The acrylamide concentrations ranged from 0.0004% to 0.4% during the incubation period, resulting in final concentrations of acrylamide from 0.47 ng/ml (6.6 nM), 80 pg/assay well, to 470 ng/ml (6.6 µM), 80 ng/assay well, in the T-cell assays. Purified Protein Derivative of tuberculin, (PPD, Statens Seruminstitut), was used at a final concentration of 12 µg/ml (2 µg/culture well).

Peripheral blood mononuclear cells (PBMC).

PBMC from healthy Danish donors were isolated from heparinized venous blood or purified from buffy coats on Lymphoprep (Nyegaard,

Oslo, Norway) using Leucosep tubes (Assaf Scientific Dev. Co. Ltd., Mevasseret-Zion, Israel) by the procedure described by the manufacturer. PBMC were cryopreserved by computer controlled gradient freezing (MIC 15, Cryoson, Middenbeemster, Holland) in liquid nitrogen at 5-8 x 10^6 cells/cryotube (Nunc, Roskilde, Denmark). Equal amounts of freezing medium (25% heat inactivated fetal calf serum (HFCS, Gibco, Paisley, U.K.), 55% RPMI 1640 (Gibco), 20% DMSO, all by volume) and cell suspension were added to the cryotubes immediately before freezing. At the day of use, cells were rapidly thawed in a 37°C waterbath, and the cells were washed once in medium + 5% HFCS, followed by 2 more washes in medium containing 15% heat inactivated normal human serum (HNHS). All media were supplemented with 20 I.U./ml. Penicillin, 20 mM Streptomycin (Gibco) and 200 mM L-glutamine (Sigma Chem. Corp. St. Louis, USA.). The donors were selected from a panel of Danish blood bank donors on basis of previous knowledge of responsiveness to soluble TT and PPD.

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Gel-separation of antigens.

Tetanus toxoid was separated by SDS-PAGE as described by Laemmli (19). Briefly, SDS-mercaptoethanol based sample buffer was added to the TT samples and boiled for 5 minutes prior to application to the gel. TT was separated in a 1 mm polyacrylamide gel, stacking gel consisting of 6% total acrylamide (T), 2.7% methylene-bis acrylamide (C) and separation gel consisting of 10% T, 2.7% C. Acrylamide solutions were all degassed prior to gelcasting and polymerized using 0.075% (v/v) tetra-methyl-ethylene-diamine (TEMED) and 0.5% (v/v) freshly made 10% ammonium persulphate. Buffers and reagents used in electrophoresis were made from molecular biology reagent grade chemicals, Sigma. Wells were washed three times in electrode buffer, before application of the samples. Electrophoresis was performed in a Hoefer SE 600 gel electrophoresis unit (Hoefer, San Francisco, CA., USA) either overnight without cooling or in 4 hours with constant current and cooling. Progress of the separation was followed by visual tracking of prestained markers (Biorad, Richmond, CA., USA). Separation of TT in agarose gels was

done in a Hoefer SE 600 unit, that never had been used for acrylamide gel electrophoresis. Briefly, the separating gel and stacking gel agarose (ProSieve, Vallensbæk Strand, Denmark) was melted and degassed by boiling for 40 minutes in a water-bath before being added to the pre-heated (60°C) gel casting stand. The sample-wells were washed in electrode buffer prior to sample application. The electrode buffer and the sample buffer used for agarose electrophoresis were the same as for the SDS-PAGE. SDS-PAGE gels were silver-stained by the procedure described by Heukeshoven (20).

Blotting to Nitrocellulose.

To locate TT, a 2 cm strip which included a marker well was cut from the gel after electrophoresis and protein stained by Coomassie blue. The remaining part of the gel was blotted onto a nitrocellulose membrane with a pore size of 0.1 μm (BA 83, Schleischer & Schüell, Dassel, Germany) as described by Kyhse-Andersen (21), in a semi-dry blot apparatus (JKA-Biotech, Værløse, Denmark). Before blotting, the membrane was washed for at least 30 minutes in sterile distilled water on a rocking table. A current of 0.8 mA/cm^2 was applied for 2 hours, and successful transfer was visualized by the complete transfer of the prestained molecular weight markers. To locate TT, two strips from the margins of the blot were cut after blotting and stained overnight in 0.1% Fount India Ink (Pelikan, Hannover, Germany) in 0.3% Tween 20/PBS. The remaining part of the blot was washed twice in 0.5 l PBS supplemented with penicillin and streptomycin (20 I.U./ml and 20 μg/ml. respectively) and air dried. The nitrocellulose paper was sterilized by irradiation (10 Krad) in a rotating ^{137}Cesium γ-emitter. Incorporation of the nitrocellulose paper into the proliferation assay was accomplished by punching out 6 mm nitrocellulose discs with sterile biopsy-punchers (Stiefel laboratorium, Offenbach a.m., Germany), after aligning of the India Ink stained blot with the sterilized blot. From each TT-blot 12 nitrocellulose discs were obtained. Control nitrocellulose discs were obtained from nitrocellulose membranes to which gels, which had been loaded with sample buffer, were blotted.

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Quantitation of ^{125}I-radio labelled Tetanus Toxoid on nitrocellulose blots.

To measure the amount of TT transferred from the different gel matrices to the nitrocellulose paper, a sample of TT was labelled with ^{125}I. TT was radiolabelled by the chloramine T iodination procedure, as described by Hunter (22). Briefly 5 µl TT (2 µg/ml), 5 µl Na-^{125}I (IMS 30, 185 MBq/50 µl, Amersham, Buckinghamshire, England) and 5 µl chloramine-T solution (6.4 µg/ml) were mixed one minute by immersion in ultrasound. Iodination was allowed to take place for 15 minutes. After iodination, the sample was desalted on a Pharmacia PD10 gelfiltration column (Pharmacia LKB Biotechnology, Uppsala, Sweden), to separate high molecular weight TT from contaminating salts. A SDS-PAGE was performed to establish the amount of labelled TT needed for visualization by autoradiography and evaluate the integrity of the labelled product. The amount of labelled TT transferred from gels to nitrocellulose was quantitated by mixing labelled TT, which had been counted in a γ-counter (Wallac, Uppsala, Sweden) with unlabelled TT and SDS-PAGE sample buffer. Then, 60 µl of the sample, corresponding to 5 µg of TT of which 0.05% was labelled, was loaded to each lane on a gel. High molecular weight prestained markers, as well as 2.5 µl of ^{14}C-labelled markers (Amersham) were run parallel to the samples. After electrophoresis a portion of the gel containing a prestained marker lane, a ^{14}C-marker lane, and two ^{125}I-TT-lanes were cut and air dried between cellophane sheets. The remainder of the gel, which also included a prestained marker lane as well as ^{14}C-labelled markers, was blotted to nitrocellulose, as decribed above. The gel which had been blotted was air dried between cellophane sheets. The pre-blot gel piece, the blotted gel and the blot were placed on film (Kodak X-omat AR, Scientific Imaging Film, New York, USA) at -85°C for 2-3 days, before developing in Kodak RP-X-OMAT Processor. The gel was then divided into 5 areas, the stacking gel area, containing high molecular weight aggregates, a 150 kDa molecular weight area, containing the TT molecule, a 50 kDa molecular weight area, the front zone and a zone between the other zones. The areas were marked using the developed film as template and cut from the gel or the paper. The pieces were placed in microtubes and

counted in a γ-counter. The quantitation of transfer of labelled TT was done twice with similar results.

Dot blots.

Dot blots of TT were made in a Biorad dot blot frame (Biorad). A pre-wetted and washed sheet of nitrocellulose was placed in the frame and 1.5 µg TT was added to each well before vacuum was applied. After two washes in PBS, the dot blot was processed as the TT-blot.

Proliferation assays.

T-cell cultures with nitrocellulose discs were done in flat bottomed 96 well microtiterplates (Nunc) with 1x 10^5 cells pr. well in a final volume of 170 µl. Before addition of PBMC and antigen to the wells, each well received 20 µl of medium in order to fix the nitrocellulose discs in the wells. Control wells received soluble antigen, medium or blank nitrocellulose. All tests were done in triplicates. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 7 days and pulsed with 20 µl of ^{3}H-thymidine (1.85 MBq/ml; NEN Research products, Boston, Mass., USA) per culture for 18-24 hours before termination of the assay. Cultures were harvested onto glass fiber filters (Skatron, Lier, Norway) and incorporation of radiolabel into DNA was determined by scintillation spectrometry (Packard Minaxi Tricarb or Packard Matrix β-counter, Groningen, The Netherlands). For each triplicate the median was recorded. A response was defined to be significant if the stimulation index (SI) $(kcpm_{stim.})/(kcpm_{control})$ >2 and the increment $(kcpm_{stim}-kcpm_{control})$ was >1 for cultures counted by liquid scintillation (Minaxi Tricarb) or >0.4 for cultures counted by the Matrix counter.

RESULTS

Separation and blotting of Tetanus toxoid in T-cell blotting.

TT was separated in both polyacrylamide and agarose gel matrices and subsequently blotted to nitrocellulose with similar results. As shown

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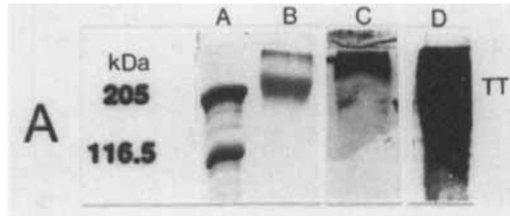

Figure 1 A. (Upper part) Lane A shows prestained high molecular weights markers. Lane B shows acrylamide gel separated TT (5 µg/lane) stained by Coomassie blue stain. Lane C shows a silver stained lane of TT (5 µg/lane) after blotting has been performed. Lane D shows an India Ink stained lane of TT (5 µg/lane) bound to nitrocellulose. Molecular weights (in kDa) and TT are indicated.

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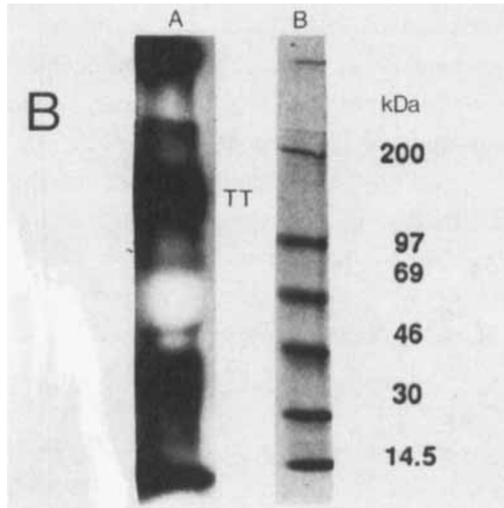

Figure 1 B. (Lower part) A section of an autoradiography of acrylamide gel separated iodinated TT (1.5 ng, lane A). Lane B shows ^{14}C-labelled markers. Molecular weights (in kDa) and TT are indicated.

with polyacrylamide gel separated TT, separation was confirmed by Coomassie blue staining of gel slices in which a 150 kDa molecular weight band was apparent (Figure 1A). The presence of a 150 kDa molecular weight band on the india ink stained nitrocellulose strips,

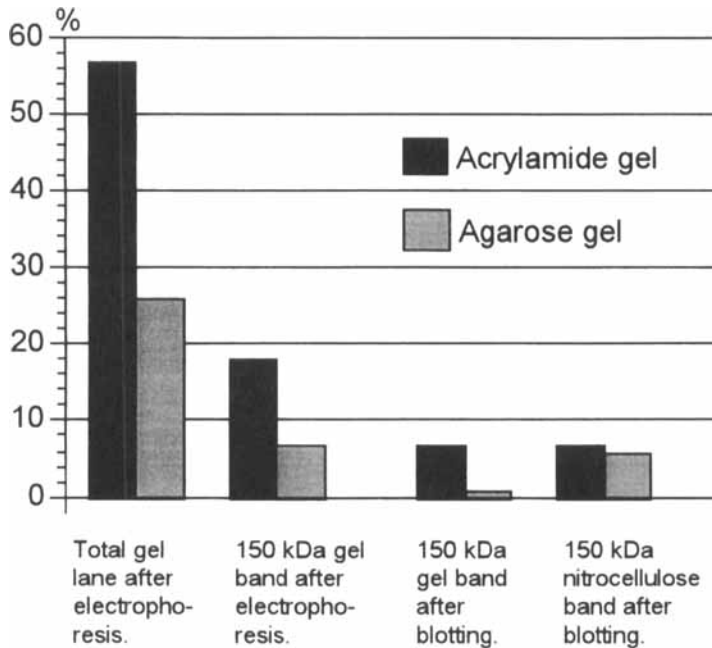

Figure 2. Transfer experiment of ^{125}I-labelled TT from either an acrylamide gel or an agarose gel to nitrocellulose. Results are shown in percent of counts per minutes of loaded sample.

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indicated that TT was transferred from the gel to the membrane (Figure 1A). The efficiency of the blotting was confirmed by staining the gel slices after blotting. These gels slices showed no trace of TT when stained by Coomassie blue, and only a faint band was observed after silver-staining (Figure 1A). The sensitivity of the silver-stain was approximately 2 ng of bovine serum albumin as a single band.

Quantitation of radiolabelled TT tranferred to nitrocellulose.

TT is degraded upon storage and by radiolabelling giving different molecular weight breakdown products, (Figure 1B). A gel filtration experiment showed that 60% of the sample was of higher molecular weight than the salt fraction, which contained 28% of the label. Five

TABLE 1

Proliferative responses of PBMC from 19 donors after stimulation with either soluble or dot blotted nitrocellulose bound TT.

Antigen	Median kcpm	Median S.I.	% Responders
Soluble TT (1.5 µg)	12.5 (3.9-80)[1]	59 (4.6-269)	100
Dotblotted TT (1,5µg)	6.7 (0.2-40)	8.0 (0.5-84)	79

1) range in brackets.

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percent of the sample was a mixture of high and low molecular weight compounds. Seven percent of the labelled TT was trapped as high molecular weight aggregates in the gel filtration matrix (data not shown). The resulting absolute transfer of 150 kDa TT to the nitrocellulose was almost identical when comparing blots from an acrylamide gel to blots from an agarose gel (Figure 2).

T-cell proliferation induced by soluble and dot blotted antigens.

T-cells were stimulated by dot blotted antigens to examine if antigen present on nitrocellulose was able to induce T-cell proliferation. Table 1 shows that even though all the donors responded to soluble TT, only 15 out of 19 donors (79%) responded to the same amount of TT blotted onto nitrocellulose.

T-cell proliferation in response to antigen separated by SDS-PAGE.

A series of experiments were performed in which TT was separated in polyacrylamide gels and blotted onto nitrocellulose. Varying amounts of TT were applied to the gels, calculated to result in the transfer of 1.5 to 20 µg TT per disc. Significant T-cell proliferation was only detected in two of the 10 donors stimulated with the high amount of TT (20 µg) (Table 2).

TABLE 2

Proliferative responses induced by soluble TT or nitrocellulose discs onto which TT had been blotted after SDS-PAGE separation.

Acrylamide gels	Median kcpm	Median S.I.	% Responders
Soluble TT (1.5 µg)¤	7.4 (1.1-35.7)[1]	43 (5.7-357)	100
1.5 µg T-cell blot *¤¤	0 (0-0.1)	1.0 (0.8-1.3)	0
20 µg T-cell blot *¤¤¤	0.3 (0-9.7)	2.3 (0-49.5)	20

1) range in brackets. * calculated amount on 6 mm nitrocellulose disc. ¤ n = 15; ¤¤ n = 5; ¤¤¤ n = 10.

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TABLE 3

Proliferative responses of PBMC from 4 donors to soluble TT and TT which had been subjected to different treatments.

Sample treatment	Median kcpm	Median S.I.	% Responders
TT; soluble	29.2 (8.7-98.4)[1]	23.0 (9.7-83)	100
TT; boiled, soluble*	58.2 (46-96.5)	53.4 (26.9-81.4)	100
TT; boiled, dotblotted**	19.1 (3.7-34.5)	38.2 (7.4-69)	100
TT; SDS/2ME, dotblot.***	14.7 (7.2-29.8)	30.4 (15.4-60.6)	100

1) range in brackets. Tetanus Toxoid was either *boiled for 5 minutes, ** boiled for 5 minutes and dot blotted or *** boiled for 5 minutes in sample buffer, and dot blotted.

To determine which step(s) in the procedure that altered the ability of TT to induce T-cell proliferation, TT was boiled and treated with electrophoresis sample buffer. Table 3 shows that neither boiling nor the addition of sample buffer to the antigen, destroyed the ability of the dotblotted TT to stimulate PBMC. These results indicated that the SDS-PAGE separation was the main step in which the stimulating capacity of TT was lost during the T-cell blot procedure. In order to determine

TABLE 4

Effect of T-cell blotted antigens on PBMC proliferation induced by soluble antigens.

Antigen	Median kcpm	Median S.I.	% Responders
Soluble TT (1.5 µg)	6.9 (5.3-8.3) [1]	31.2 (27.5-38)	100
Soluble PPD	76.7 (46.7-103.9)	209.9 (130.2-445)	100
TT + Tcb (20 µg TT)[2]	2.9 (2.2-21.2)	14.0 (7.8-17.3)	100
PPD + Tcb (20 µg TT)	78.2 (6.5-98.5)	136.9 (33.5-493.5)	100

1) range in brackets, n = 4. 2) SDS-PAGE T-cell blot with 20 µg of TT pr. nitrocellulose disc was added to the cultures stimulated with soluble antigen.

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TABLE 5

T-cell proliferation induced by agarose T-cell blotting.
(Results are given as percentage of the response to solubleTT, 1.5 µg)

Agarose gels	Median kcpm (%)	Median S.I. (%)	% Responders
3.3 µg TT/disk[¤]	18.9 (10.0-38.8)	44.9 (10.6-58.6)	57
5-7 µg TT/disk[¤¤]	65.9 (10.3-400.0)	30.4 (12.1-306.3)	80
9-10 µg TT/disk[¤¤]	22.7 (7.3-151.4)	3.8 (0.5-77.0)	50

1) range in brackets. • Negative T-cell blots not included in figures for kcpm; ¤ n =7 ; ¤¤ n =10.

whether the TT bound on the nitrocellulose after T-cell blotting was toxic to the cells in culture, we activated the PBMC with soluble antigens in the presence of nitrocellulose discs. Table 4 shows that the antigen bound to nitrocellulose did not affect the soluble PPD response, and only slightly affected the soluble TT response.

To further substantiate that the SDS-PAGE separation played a major role in the inability of TT to stimulate PBMC cultures after T-cell blotting,

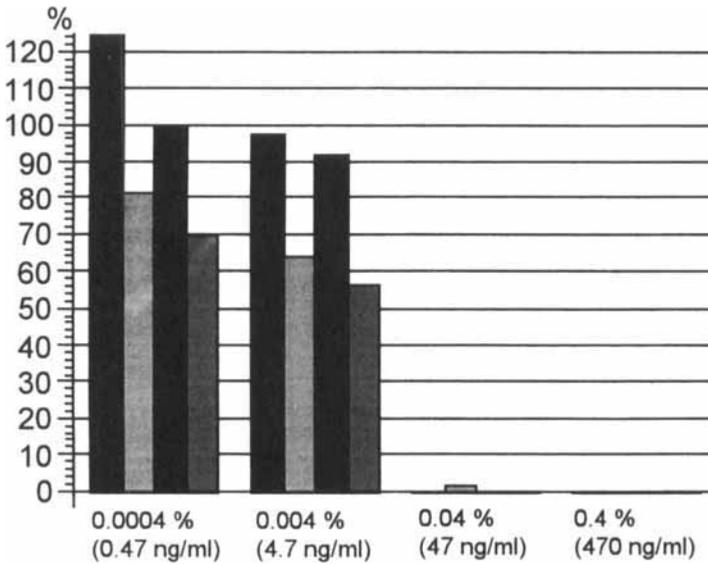

Figure 3. The effect of preincubating varying amounts of acrylamide monomer with TT before adding the mixture to the T-cell proliferation assay. Proliferative response in percent of response to soluble TT. Each column represents one donor.

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we changed the matrix of the gel to agarose, while all other steps in the T-cell blot procedure were left unchanged. Table 5 shows that most donors responded to TT bound to nitrocellulose after blotting was performed with SDS-AGE separation, although the proliferative responses were lower than those induced by soluble TT.

T-cell proliferation to TT coincubated with varying concentrations of acrylamide monomer.

To establish if monomeric acrylamide inhibited the proliferative response, varying concentrations of acrylamide was added to TT stimulated PBMC cultures. Figure 3 shows that monomeric acrylamide is a potent inhibitor of T-cell proliferation.

DISCUSSION

Screening for T-cell antigens in complex antigen mixtures is a tedious process, that usually requires protein purification by traditional protein fractionation techniques. T-cell blotting as a method of screening for T-cell antigens, has the theoretical advantage of an excellent separation of antigens combined with a relatively low antigen requirement. The T-cell blotting method has been used for identification of T-cell antigens from various sources. However, early reports have described low T-cell proliferative responses to antigens bound to nitrocellulose after SDS-PAGE separation compared to the responses induced by unseparated or dotblotted antigen (4,512,17).

In this paper, the T-cell blotting method was evaluated by comparing the ability of a model antigen to induce proliferation of lymphocytes in the soluble form and after SDS electrophoresis and blotting. TT was chosen as a model antigen because of its well defined mobility in the SDS-PAGE, and the availability of PBMC responding to TT in vitro. We found that TT blotted to nitrocellulose after SDS-PAGE separation was unable to induce proliferation of PBMC from the majority of the donors, even though the PBMC responded to soluble TT.

The hypothesis that polyacrylamide interfered with the stimulatory capacity of TT, was supported by experiments showing that TT could induce proliferation in T-cell blotting experiments, if the polyacrylamide gel matrix was replaced by an agarose gel matrix (Table 4). The better performance of SDS-AGE separated antigen was not due to a higher concentration of TT on the nitrocellulose membrane after SDS-AGE than after SDS-PAGE separation (Figure 2).

To investigate the mechanisms underlying the lack of response to TT in the T-cell blotting experiment, we tested the stimulatory capacity of TT obtained at different stages of the T-cell blotting procedure. We first tested whether TT bound to nitrocellulose after dot blotting was able to stimulate the PBMC. The dot blotted TT induced proliferation in most donors, indicating that TT bound to nitrocellulose is accessible to the PBMC (Table 1). We then tested whether the SDS-treatment interfered with the ability of TT to induce proliferation. This did not seem to be the

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case, since proliferative responses induced by dot blots of TT, which had been boiled in sample buffer with reducing agent (2-Mercaptoethanol), did not differ from the ordinary dot blot results (Table 3). These data suggested that the missing proliferative responses to TT in the T-cell blot assay could not be explained by destruction of TT quarternary structure by the treatment in sample buffer or inaccesibility of T-cell epitopes on the nitrocellulose paper. This implied that the ability of TT to induce proliferation was lost during the separation in the polyacrylamide gels.

We found that monomeric acrylamide in concentrations as low as 4.7 ng/ml (equaling 6.6 nM) inhibited T-cell proliferation (Figure 3). Even under optimal polymerization of a 10% polyacrylamide gel matrix, only up to 96% of monomeric acrylamide is polymerized, leaving a 50-60 mM unpolymerized monomeric acrylamide in the gel (23). There is ample evidence that monomeric acrylamide can bind to protein. Binding of ^{14}C-acrylamide to microtubuli and neurofilaments was demonstrated, even at monomeric acrylamide concentrations as low as 0.1-30 μM (24). Moreover, Narasimhulu (25) and Tallmagde et al. (26) have shown that acrylamide binds to tryptophane; Örstan and Gafni (27) have demonstrated that acrylamide binds to cysteine residues in glyceraldehyde-3-phosphate dehydrogenase, while Krysteva et al. (28) exploited the binding capabilities of acrylamide to study enzymes immobilized onto an acrylamide containing membrane. Finally, Geisthardt and Kruppa (29) reported that a change in the mobility of bovine serum albumin in SDS-PAGE was due to binding of monomeric acrylamide to the ε-amino groups of the lysine residues. TT contains more than 8% lysine (30) thus making TT highly susceptible to acrylamide alkylation during electrophoresis. Migration of TT and other proteins through the acrylamide gel electrophoresis system might therefore induce varying degrees of acrylamide alkylation, depending on the contents of reactive amino acid residues in the separated proteins. Molecules containing no or only very few reactive amino acids would therefore probably be subject to less monomeric acrylamide alkylation than molecules containing more reactive amino acid residues. That this can be the case is demonstrated by Bonaventure et al. (31), studying differential monomeric acrylamide alkylation of human and rat hemoglobin α- and β-chains after SDS-PAGE,

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showing up to 100% alkylation of the rat β-chain, as measured by electrospray ionization mass spectrometry. The same study showed that in the volume of 2.5 ml polymerized 5% acrylamide in a rod gel, sufficient unreacted monomeric acrylamide was present to alkylate 6 mg of the rat hemoglobin β-chain.

Hershko (32) has suggested that lysine plays a crucial role in antigen degradation during processing of endogenous antigens. Degradation of intracellular proteins is preceeded by covalently binding of ubiquitin to the ε-amino group of lysine residues, thus tagging these proteins for degradation. A similar interaction of the ε-amino groups of lysine with acrylamide could inhibit this tagging by ubiquitin or similar molecules, and subsequently prevent degradation and presentation of the antigen. Although the new agarose compound used in this study is not as manipulative as the standard polyacrylamide in terms of altering the gel poresizes or making gradient gels, this compound is, jugded from the experiments performed, a valid separation matrix. However, it is our experience that separation of low molecular weight antigens (less than approximately 20 kDa) is not efficient.

In conclusion, our data suggest that T-cell proliferation in functional T-cell assays may be hampered by the binding of monomeric acrylamide, to antigens separated by SDS-PAGE, interfering by inhibiting or lowering T-cell proliferation. Absence of T-cell proliferation when doing SDS-PAGE T-cell blots should therefore be interpreted with caution.

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