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Immunology

Two genetically identical antigen-presenting cell clones display heterogeneity in antigen processing

(immunodominant T-cell determinant/T-cell activation)

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ABSTRACT **Evidence from various antigen systems suggests that antigen processing can be one factor that determines the repertoire of immunogenic peptides. Thus, processing events may account for some of the disparity between the available and expressed helper T-cell repertoires. In this report, we demonstrate that the immunodominant T-cell determinant in ovalbumin [p323–339; ovalbumin-(323–339) heptadecapeptide] is processed differently by two genetically identical antigen-presenting cell lines, M12 and A20. The ovalbumin-specific T-cell–T-cell hybridomas, DO-11.10 and 3DO-54.8, were used to detect processed antigen. These T–T hybridomas have different fine specificities for the p323–339 determinant. A20 cells presented native ovalbumin well to both T–T hybridomas, whereas M12 cells presented native ovalbumin well to 3DO-54.8 but very inefficiently to DO-11.10. M12 and A20 cells effectively stimulated both T–T hybridomas with the same concentrations of the immunogenic synthetic peptide p323–339. Therefore, M12 cells and DO-11.10 can interact with each other, and both T–T hybridomas have similar sensitivities for the same immunogenic peptide. We conclude that genetically identical antigen-presenting cells can display heterogeneity in the fine processing of an immunodominant T-cell determinant, and synthetic model peptides that represent the minimal stimulatory sequence of a T-cell determinant are not necessarily identical to the structure of *in vivo* processed antigen. Heterogeneity in antigen processing by individual antigen-presenting cells would serve to increase the repertoire of immunogenic peptides that are presented to T cells.**

Helper T lymphocytes recognize foreign antigen in association with class II major histocompatibility complex molecules (Ia) on the surface of antigen-presenting cells (APC) (1, 2). Prior to recognition, native antigen is internalized and processed by the APC (3, 4). The conversion of a native, nonstimulatory protein to an immunogenic form is commonly referred to as antigen processing. Treating the APC with aldehydes (4, 5), lysosomotropic chemicals (6, 7), or specific protease inhibitors (8, 9) can block antigen processing without affecting the function of Ia molecules on the APC surface. *In vitro* proteolytic or chemical cleavage of proteins can generate peptide fragments that function as antigens with processing-inactive APC (3, 9, 10). Thus, it is generally accepted that cellular processing of a protein antigen occurs in a lysosomal and/or endosomal compartment and produces immunogenic peptide fragments. Subsequent to antigen processing, the antigen is expressed on the APC surface, where it physically interacts with an Ia molecule (11–14) and possibly with membrane lipids (15, 16). The formation of a trimolecular complex consisting of Ia, processed antigen, and the T-cell receptor is required for antigen-specific activation of the helper T lymphocyte. At the present time, functionally relevant *in vivo* processed antigen on the surface of an APC can only be detected by measuring the stimulation of antigen-specific T cells.

Much of our present understanding of the interaction between the T-cell receptor, antigen, and the Ia molecule has been gained by using synthetic peptides to analyze the requirements for T-cell activation. Series of sequentially truncated synthetic peptides have been used to define the minimal stimulatory length of several T-cell determinants (17–20). Residues in a T-cell determinant that interact specifically with the T-cell receptor or Ia molecule have been identified by determining the effects of single amino acid substitutions on T-cell stimulation and on binding to Ia molecules (20, 21). Since *in vivo* processed antigen has not been isolated and structurally defined, it is not known whether *in vivo* processing produces peptides with a minimal stimulatory sequence of amino acids.

There are numerous examples in which the T-cell response utilizes only a selected number of T-cell determinants in a multideterminant antigen (22, 23). Several reports suggest that antigen processing can be a factor that determines the repertoire of immunogenic peptides available for interaction with Ia molecules (19, 24–27). In support of this argument, we provide evidence that the immunodominant T-cell determinant in native ovalbumin is processed differently by two genetically identical APC clones. The product of this differential processing is detected by two T-cell hybridomas, which have different fine specificities for the immunodominant determinant.

MATERIALS AND METHODS

Reagents. Crystallized chicken ovalbumin was purchased from ICN Immunobiologicals, Lisle, IL, and dissolved in phosphate-buffered saline at 10 mg/ml. The synthetic peptide p323–339 [ovalbumin-(323–339) heptadecapeptide] with an additional single tyrosine residue at the carboxyl terminus was synthesized and provided by G. Matsueda (Massachusetts General Hospital, Boston, MA). HPLC analysis and partial amino acid sequence analysis indicated that the synthetic peptide was >90% pure. The biological properties of this peptide have been described (14, 28). Ovalbumin was digested with trypsin as described (10).

Cell Lines. The T-cell–T-cell hybridomas DO-11.10.S4.4 [DO11.10; BALB/c (anti-ovalbumin–I-A^d)–BW5147 thymic lymphoma], 3DO-54.8 [BALB/c (anti-ovalbumin–I-A^d)–BW5147], and 8DO-51.15 [BALB/c (anti-ovalbumin–I-A^d)–BW5147] were provided by J. Kappler and P. Marrack (National Jewish Hospital, Denver, CO) and have been described (10, 20). These T-cell hybridomas are activated to produce the lymphokine interleukin 2 (IL-2) upon recognition of the appropriate antigen on the surface of Ia-bearing accessory

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Abbreviations: APC, antigen-presenting cell(s); IL-2, interleukin 2; p323–339, ovalbumin-(323–339) heptadecapeptide.

cells. The A20.2J (A20) cell line (ref. 29; Ia-positive BALB/c lymphoblastoid B cells) was provided by J. Kappler and P. Marrack. The M12.4.1 (M12) cell line (ref. 30; Ia-positive BALB/c lymphoblastoid B cells) was provided by L. Glimcher (Harvard School of Public Health, Boston, MA). All cell lines are passaged *in vitro* in Dulbecco's modified Eagle's medium with 4.5 g of glucose per liter supplemented with 5% (vol/vol) heat-inactivated fetal calf serum, 4 mM L-glutamine, and 100 units of penicillin, 100 μg of streptomycin, and 0.25 μg of fungizone per ml. Media and supplements except fetal calf serum were purchased from Irvine Scientific.

Cell Culture. Culture medium was RPMI 1640 (Irvine Scientific) with 10% fetal calf serum and was supplemented as indicated above along with 20 mM Hepes (Irvine Scientific), 50 μM 2-mercaptoethanol, and 1 mM nonessential amino acids (Irvine Scientific). Cultures containing 10^5 T-cell hybrids per well with or without a source of APC (10^5 per well) in the presence or absence of antigen were brought to a final volume of 200 μl in flat-bottom microculture plates. The T-cell hybridoma cultures were incubated at 37°C for 18–24 hr, after which time 100 μl of supernatant was removed, frozen, or exposed to γ radiation (8000 rads; 1 rad = 0.01 Gy) and then assayed for IL-2 content. The IL-2-dependent cell line HT-2 was used to assay for IL-2 as described (31). In some experiments APC were exposed to antigen by incubating cells (7.5×10^5 per ml) with 1 mg of ovalbumin per ml for 18 hr at 37°C. The cells were then extensively washed with ice-cold phosphate-buffered saline (PBS) and either kept alive or incubated with 1% paraformaldehyde for 10 min at room temperature. The fixed cells were washed free of fixative with ice-cold phosphate-buffered saline. APC not incubated with ovalbumin were fixed with paraformaldehyde as described above.

RESULTS

Presentation of Ovalbumin by Two BALB/c Lymphoblastoid B-Cell Lines. While testing various I-A^d-bearing APC for their ability to present ovalbumin to the T–T hybridomas DO-11.10 and 3DO-54.8, we observed that M12 cells presented ovalbumin well to 3DO-54.8 but poorly to DO-11.10 (Fig. 1*A*). The results in Fig. 1*A* are representative of 9 of 11 experiments. In the remaining 2 experiments, the dose–response curves still differed markedly, but DO-11.10 did produce higher levels of IL-2 when ovalbumin was at 400 and 800 μg/ml with M12 cells.

In the same experiment, A20 cells presented ovalbumin well to both T–T hybridomas (Fig. 1*B*). In each of the 11 experiments mentioned above, the dose–response curves of both T–T hybridomas with A20 cells did not differ from one another by more than a 4-fold concentration of ovalbumin. Despite this variation in responsiveness of the T–T hybridomas, when compared in the same experiment, M12 cells presented ovalbumin well to 3DO-54.8 but inefficiently to DO-11.10, while A20 cell presented ovalbumin well to both T–T hybridomas. We wish to emphasize that the results in Fig. 1 *A* and *B* were obtained in the same experiment. The ability of DO-11.10 to respond to ovalbumin presented by A20 cells demonstrates that DO-11.10 was functionally active. The observation that M12 cells presented ovalbumin better to 3DO-54.8 than to DO-11.10 was unexpected because both T–T hybridomas recognize the epitope contained within the tryptic ovalbumin fragment p323–339.

A second experimental protocol was used to further confirm that M12 cells are deficient in their ability to present ovalbumin to DO-11.10. M12 and A20 cells were incubated with ovalbumin at 1 mg/ml for 18 hr to allow uptake, processing, and display of processed antigen on the cell surface. The cells were then washed extensively, and each cell line was divided into two groups; one group was kept alive and the other was fixed with 1% paraformaldehyde. Treatment with paraformaldehyde prevents further antigen processing (4, 5). Since the APC were incubated with antigen in the absence of a T–T hybridoma, a lack of IL-2 production could not be caused by a T–T hybridoma inhibiting a step required for antigen processing. APC that were preincubated with ovalbumin at 1 mg/ml were then assayed for their ability to stimulate DO-11.10 and 3DO-54.8. The results in Fig. 2 are representative of three experiments. Live (Fig. 2*A*) and fixed (Fig. 2*B*) M12 cells that were preincubated with ovalbumin were unable to stimulate DO-11.10, whereas, both groups of M12 cells stimulated 3DO-54.8 in a dose-dependent manner. As part of the same experiment, live (Fig. 2*C*) and fixed (Fig. 2*D*) A20 cells that were preincubated with ovalbumin stimulated both T–T hybridomas in a similar dose-dependent manner. The dose–response curves for DO-11.10 and 3DO-54.8 with either group of A20 cells never differed by more than 4-fold in APC number. Thus, when both T–T hybridomas have similar dose–responses to A20 cells preincubated with ovalbumin, M12 cells, also preincubated with ovalbumin, are severely deficient in their ability to stimulate DO-11.10 but not 3DO-54.8. By ruling out the possibility that DO-11.10 could have an effect on the processing and presentation of ovalbumin, the results in Fig. 2 further confirm that M12 cells are deficient in their ability to present ovalbumin to DO-11.10.

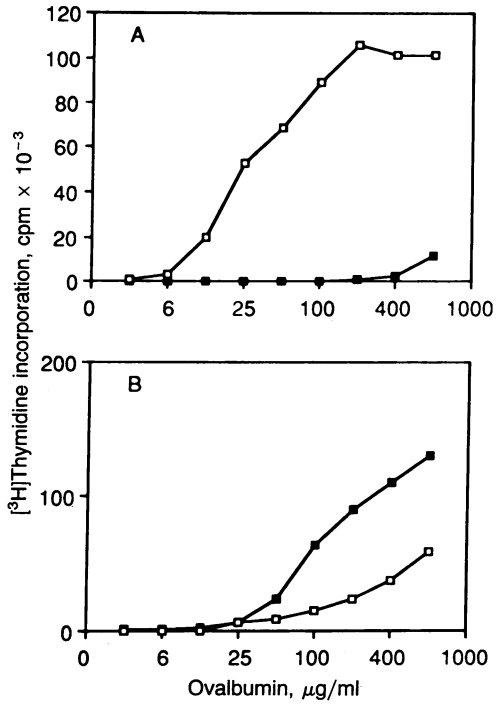

FIG. 1. Differential processing of ovalbumin by two BALB/c lymphoblastoid B-cell lines. Duplicate microcultures (200 μl) with the indicated concentration of ovalbumin were prepared with the T-cell hybridomas (10^5 cells) DO-11.10 (■) and 3DO-54.8 (□) and either 10^5 M12 APC (*A*) or 10^5 A20 APC (*B*). Cultures were incubated for 18–24 hr at 37°C, after which 100 μl of supernatant was removed, x-irradiated or frozen, and assayed for IL-2 content by measuring incorporation of [^{3}H]thymidine by IL-2-requiring HT-2 cells. The results of a single experiment are shown (see *Results* section).

M12 Cells Present the Synthetic Ovalbumin Peptide p323–339 to DO-11.10. Previous studies have shown that the synthetic ovalbumin peptide p323–339, in association with I-A^d, can stimulate DO-11.10 and 3DO-54.8 (20, 28). In contrast to native ovalbumin, p323–339 does not require processing because the peptide can be presented by alde-

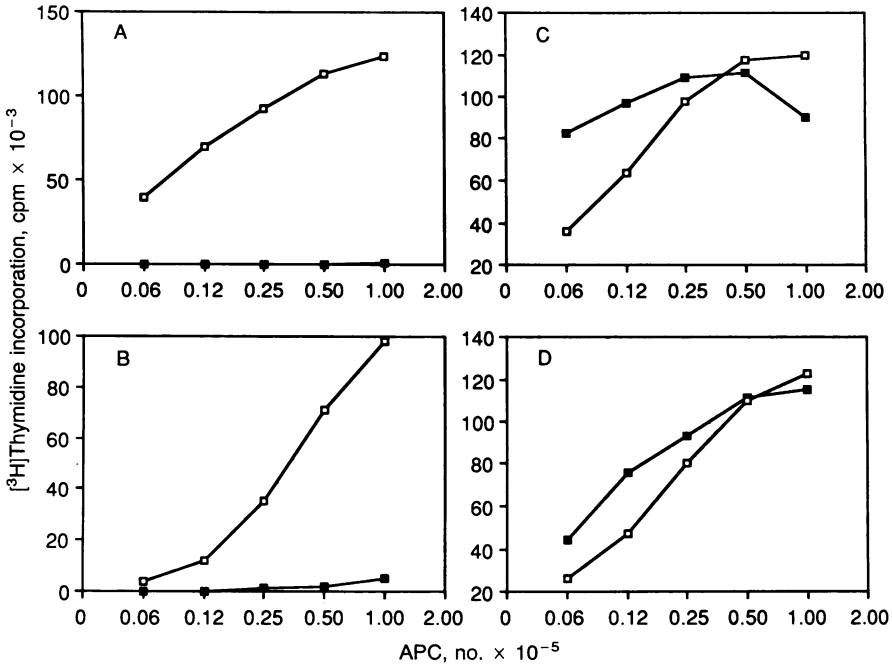

FIG. 2. Stimulation of the T-cell hybridomas DO-11.10 and 3DO-54.8 by APC preincubated with ovalbumin. M12 (*A* and *B*) and A20 (*C* and *D*) APC were preincubated with ovalbumin (1 mg/ml) for 18 hr at 37°C, washed extensively, and then kept alive (*A* and *C*) or fixed (*B* and *D*). Duplicate microcultures (200 μl) were prepared with 10^5 DO-11.10 (■) or 10^5 3DO-54.8 (□) and the indicated number of treated APC. The microcultures were incubated, and the supernatants were assayed for IL-2 content as described in the legend to Fig. 1. The results are representative of three experiments.

hyde-fixed APC. We used the peptide p323–339 to determine whether fixed M12 cells could present an appropriate immunogenic peptide to DO-11.10. The amount of p323–339 that is required to stimulate each T–T hybridoma is also a specific indication of the sensitivity of the T–T hybridomas for processed antigen. Fig. 3 shows the results from two of three experiments in which the stimulation of both T–T hybridomas in the presence of fixed APC and p323–339 was measured. M12 (Fig. 3 *A* and *C*) and A20 cells (Fig. 3 *B* and *D*) in the presence of p323–339 stimulated DO-11.10 and 3DO-54.8 in a dose-dependent manner. Similar results were obtained with trypsin-digested ovalbumin as the antigen in culture (unpublished data). The dose–response curves in Fig. 3 illustrate several important points. (*i*) This experiment confirms that

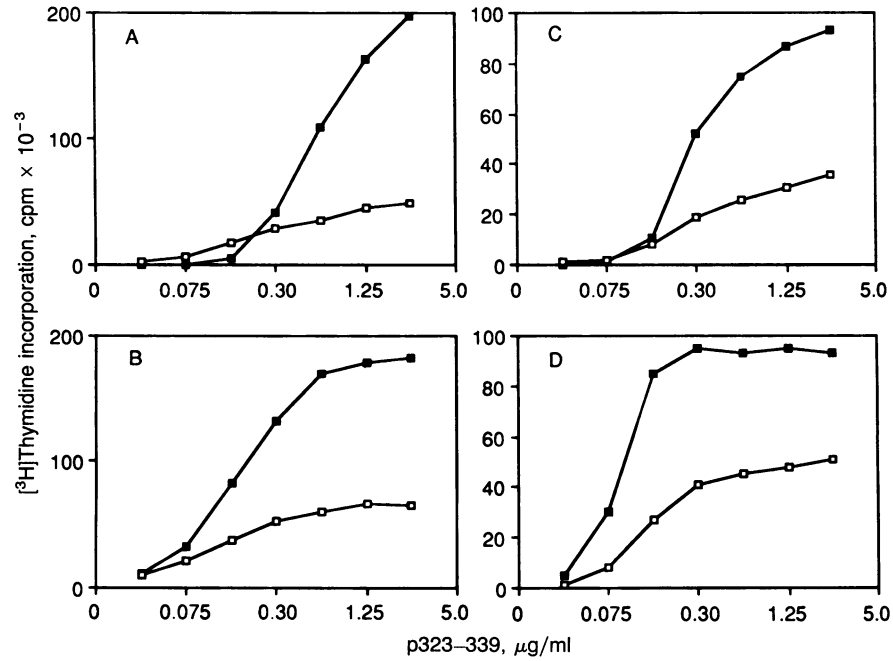

FIG. 3. Dose–responses of the T-cell hybridomas DO-11.10 and 3DO-54.8 to the synthetic ovalbumin peptide p323–339. Duplicate microcultures (200 μl) with the indicated concentration of p323–339 were prepared with the T-cell hybridomas (10^5 cells) DO-11.10 (■) and 3DO-54.8 (□) and either 10^5 fixed M12 APC (*A* and *C*) or 10^5 fixed A20 APC (*B* and *D*). The microcultures were incubated, and the supernatants were assayed for IL-2 content as described in the legend to Fig. 1. The results in *A* and *B* and those in *C* and *D* were from two of three experiments. In the presence of ovalbumin at 800 μg/ml, the responses for all four combinations of APC plus T-cell hybridoma were <1000 cpm. The data in *A* and *B* and in Fig. 1 are derived from the same experiment.

the immunogenic peptide p323–339 stimulates both DO-11.10 and 3DO-54.8, as expected. (*ii*) M12 cells stimulated both T–T hybridomas with the immunogenic peptide p323–339 (Fig. 3 *A* and *C*). In fact, DO-11.10 released more IL-2 than did 3DO-54.8. Thus, the inability of M12 cells to present native ovalbumin to DO-11.10 is not due to an inability of M12 cells and DO-11.10 to interact with each other. (*iii*) M12 cells effectively stimulated both T–T hybridomas with the same concentrations of the immunogenic peptide p323–339 (Fig. 3 *A* and *C*). This was not the case when native ovalbumin was processed *in vivo* (compare Fig. 3*A* with Fig. 1*A*, which were derived from the same experiment). Therefore, we conclude that the two T–T hybridomas have similar sensitivities to the peptide p323–339 and that the predominant form of processed ovalbumin produced by M12 cells is not the peptide p323–339. (*iv*) Since A20 cells presented native ovalbumin and p323–339 similarly to both T–T hybridomas (compare Fig. 3*B* with Fig. 1*B*, which were derived from the same experiment), we conclude that A20 and M12 cells process native ovalbumin differently.

DISCUSSION

The results presented herein illustrate that clonal differences in the processing of an immunodominant T-cell determinant can be found in genetically identical APC.

M12 cells either in culture with ovalbumin or preincubated with ovalbumin stimulated 3DO-54.8 better than DO-11.10. In contrast, this difference in the presentation of native ovalbumin was not observed when A20 cells were used as APC. The ability of M12 cells to stimulate DO-11.10 in the presence of p323–339 shows that DO-11.10 can recognize an appropriate immunogenic peptide in association with I-A^d on the surface of M12 cells. This result implies that M12 cells do not lack an accessory molecule and do not express an inhibitory membrane structure that specifically prevents an interaction with DO-11.10. 3DO-54.8 and DO-11.10, in the presence of M12 or A20 cells, produced similar dose–responses to p323–339 even in the experiments where both T-cell hybridomas responded differently to native ovalbumin processed by M12 cells. Therefore, M12 cells express normal Ia molecules, and both T-cell hybridomas have similar abilities to detect and respond to the same specific immunogenic peptide on the surface of M12 cells. In summary, the decreased ability of M12 cells to process and present ovalbumin to DO-11.10 cannot be attributed to DO-11.10 inhibiting M12 cells from processing ovalbumin, a lack of interaction between DO-11.10 and M12 cells, or a marked difference in sensitivity between DO-11.10 and 3DO-54.8. We conclude from the different responses of DO-11.10 and 3DO-54.8 to ovalbumin processed by M12 and A20 cells, that the immunodominant T-cell determinant in ovalbumin localized to the tryptic fragment p323–339 is processed differently by A20 and M12 cells. The manner in which M12 cells process ovalbumin markedly favors the stimulation of 3DO-54.8. Also, the responses of 3DO-54.8 and 8DO-51.15 to ovalbumin processed by A20 and M12 cells are very similar (unpublished data).

Our results do not define the precise molecular basis for the processing difference between M12 and A20 cells. As initially described by Shimonkevitz *et al.* (28), DO-11.10 and 3DO-54.8 are stimulated by the ovalbumin heptadecapeptide p323–339 in association with I-A^d. However, their fine specificities are different because the truncated ovalbumin-(323–336) tetradecapeptide, p323–336, stimulates 3DO-54.8 but not DO-11.10. 3DO-54.8 responds in a similar dose-dependent fashion to both peptides (28). This result and the results of competitive binding experiments with purified I-A^d (20) support the conclusion that p323–339 and p323–336 have an identical capacity to bind to I-A^d. A more extensive analysis of the p323–339 peptide has been described by Buus and co-workers (20, 32). Stimulation of the T-cell hybridomas DO-11.10, 3DO-54.8, and 8DO-51.15 has revealed three different patterns of recognition of the p323–339 sequence. Stimulation of DO-11.10, 3DO-54.8, and 8DO-51.15 required respectively the ovalbumin p327–337 undecapeptide, p326–336 undecapeptide, and p323–336 tetradecapeptide. Based on these observations, there is a possibility that M12 cells may largely produce an immunogenic form of ovalbumin with a C-terminal glutamic acid residue (Glu-336). Alternatively, it is also possible that the fragment of ovalbumin produced by M12 contains the 323–339 heptadecapeptide sequence as well as an epitope-specific hindering structure that interferes with the interaction between the peptide and the T-cell receptor on DO-11.10 but not 3DO-54.8. The presence of such a hindering structure has been suggested as the molecular basis for the conversion from a nonstimulatory (p60–104) to a stimulatory (p66–104) fragment of pigeon cytochrome *c* (33). In this case, Lys-99, which is one residue that is believed to interact with the T-cell receptor, has the potential to form an electrostatic interaction with Glu-61. Disruption of this interaction by removing Glu-61 would uncover and allow Lys-99 to interact with the T-cell receptor. A second mechanism by which amino acid residues that are outside the immunodominant epitope could influence antigen presentation would be if they interfered with antigen binding to Ia molecules. Brett *et al.* (24) have recently reported an apparent example of this effect. A related phenomenon has also been reported for the processing and presentation of an immunogenic peptide (25). This second mechanism is unlikely to account for our results because M12 cells present ovalbumin to 3DO-54.8, and I-A^d is the restriction molecule for both DO-11.10 and 3DO-54.8. Results in several reports suggest that the products of antigen processing can differ when the structural form of an immunogen is changed (i.e., native vs. denatured or fragmented) (19, 34, 35). Although our results do not argue against this possibility, differential processing of ovalbumin in our experiments occurred without prior *in vitro* modification of the native molecule. Since A20 and M12 cells are genetically identical, processing mechanisms rather than the Ia molecule had the more dominant role in determining the primary structure of the immunogenic fragment of ovalbumin. Even though we cannot identify the precise molecular species of ovalbumin produced and displayed by M12 cells, our present results do allow us to conclude that a large majority of the immunogenic fragments produced by M12 and recognized by 3DO-54.8 cannot have the exact amino acid sequence of p323–339. Hence, in this case, synthetic peptides that represent the minimal stimulatory sequence of a T-cell determinant only approximate what is made *in vivo*. A similar conclusion can be drawn from the findings of Brett *et al.* (24) and Fox *et al.* (25).

Our results extend previous studies that have shown heterogeneity in the ability of APC to present antigen. While testing a number of lymphoblastoid B-cell lines for their ability to present different antigens, Walker *et al.* (36) observed that three cell lines presented keyhole limpet hemocyanin (KLH) to one particular KLH-specific T-cell hybridoma and not to another. The basis for this phenomenon was not defined, although the authors suggested that these cell lines could possibly differ in their ability to process and display a particular antigenic epitope. A differential ability to process various distinct antigens has been observed with Ia-transfected L-cell fibroblasts. The L-cell transfectants were capable of presenting KLH, but the same L-cell transfectants failed to present native ovalbumin and hen egg lysozyme (HEL) to antigen-specific T-cell clones and T-cell hybridomas (37, 38). One L-cell transfectant that was deficient in its ability to present HEL, did stimulate a HEL-specific T-cell line and T-cell hybridoma with the appropriate

immunogenic peptide (39). These results suggested that a fibroblast might be deficient in its ability to process some antigens. We report here that genetically identical APC clones show differences in the fine specificity of antigen processing for the same T-cell determinant. It is also noteworthy that these clones are derived from the same lineage of cells.

Within any given antigen, there will be only a limited number of potential peptides that can associate with Ia molecules and become available for T-cell recognition. There is accumulating evidence that antigen processing can be a factor that determines the repertoire of peptides that is available for interaction with Ia molecules. As discussed above, a potential T-cell determinant may not be expressed because *in vivo* processed antigen contains a hindering structure that affects the interaction with an Ia molecule or the T-cell receptor or both. Our findings with M12 cells constitute a further example of how processing events can affect the repertoire of peptides that are available for interaction with Ia and subsequent immune recognition. In this context, our results have a further implication that should be noted. If individual clones of APC, such as M12 and A20, process native antigen differently, then APC heterogeneity may exist *in vivo*. Heterogeneity in antigen processing would generate a greater diversity of peptides and thus increase the antigenic diversity available for immune recognition.

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