Antigen presentation of egg-white lysozyme but not of ribonuclease A is augmented by the major histocompatibility complex class II-associated invariant chain

ARTICLE *in* EUROPEAN JOURNAL OF IMMUNOLOGY · MAY 1991

Impact Factor: 4.03 · DOI: 10.1002/eji.1830210524 · Source: PubMed

7 AUTHORS, INCLUDING:

José Moreno
Hospital Juárez de México, Secretaría de Sal…
54 PUBLICATIONS **871** CITATIONS
SEE PROFILE

Frank Momburg
German Cancer Research Center
149 PUBLICATIONS **8,837** CITATIONS
SEE PROFILE

Luciano Adorini
Intercept Pharmaceuticals
354 PUBLICATIONS **18,231** CITATIONS
SEE PROFILE

Farsin Nadimi, José Moreno▼, Frank Momburg, Andreas Heuser, Serge Fuchs○, Luciano Adorini○ and Günter J. Hämmerling

German Cancer Research Center, Institute for Immunology and Genetics, Heidelberg and Sandoz Pharma Ltd.○, Preclinical Research, Department of Immunology, Basel

Antigen presentation of hen egg-white lysozyme but not of ribonuclease A is augmented by the major histocompatibility complex class II-associated invariant chain*

The influence of the class II-associated invariant chain (Ii) on the presentation of the protein antigens hen egg-white lysozyme (HEL) and ribonuclease A (RNase) was investigated. For this purpose the Ii$^-$ rat-2 fibroblasts were transfected with I-A^k genes with or without Ii. Transfectants expressing Ii were superior in the presentation of the complete HEL protein to a panel of I-A^k-restricted T hybridomas characterized by distinct specificities for different HEL peptides and by different sensitivities to antigen concentration. There appeared to be a correlation between the antigen-presenting capacity and the amount of Ii, in that transfectants expressing large amounts of Ii were the best antigen presentors. The presentation of synthetic HEL peptides was not infuenced by Ii.
In contrast to the findings with HEL, the presentation of RNase by the same set of transfectants was clearly independent of Ii. Both antigens, HEL and RNase, required processing in the chloroquine-sensitive compartment. However, only the presentation of HEL but not of RNase could be efficiently blocked by brefeldin A. These data confirm that presentation of HEL depends on *de novo* synthesized class II molecules, whereas the presentation of RNase seems to be predominantly mediated by a pool of pre-existing class II molecules whose interaction with endocytosed antigen does not depend on Ii. These results suggest different mechanisms for the presentation of HEL and RNase and they raise the possibility that different antigens intersect the class II pathway at distinct intracellular locations.

1 Introduction

It is now well established that the TcR recognizes peptides derived from degraded proteins which are bound to the cleft of MHC molecules [1–3]. MHC class I molecules bind preferentially endogenously derived peptides, probably shortly after biosynthesis of class I in the endoplasmic reticulum (ER). The complex consisting of class I, β_2-microglobulin and peptide is then transported to the cell surface where it can be recognized by class I-restricted CD8$^+$ cells. However, there are also exceptions to this rule as documented by the experimental introduction of soluble OVA into the biosynthetic pathway of class I [4].

In contrast to class I, MHC class II molecules present antigens via the endocytic/endosomal pathway [2]. The respective antigens, usually exogenous antigens, are internalized by endocytosis and degraded by proteases in the endosomal compartment, where the peptides are met and bound by class II molecules and the resulting complex transported to the cell surface to be presented to CD4$^+$ cells. Agents, such as chloroquine, which abolish the low pH in endocytic structures including endosomes, interfere with antigen presentation by class II but not class I [5].

Although the majority of antigens which are presented by the endocytic pathway are exogenous proteins, there is now increasing evidence that endogenous cellular proteins can also be presented by class II molecules, for example viral proteins in infected cells [6–10]. In the vast majority of these cases endosomal activity seems to be required. Various mechanisms could be involved in the class II-restricted presentation of endogenous antigens, such as endocytosis of membrane proteins, release of cytosolic proteins followed by re-uptake via the endocytic-exogenous pathway, or intracellular translocation of endogenous proteins to the endosomal coimpartment by as yet unknown mechanisms. There exist only very few examples suggesting that class II can present endogenous antigens via the biosynthetic pathway and independent of endosomal activity ([9], Moreno et al., submitted).

The existence of two distinct pathways (biosynthetic *vs.* endocytic pathway) for antigen presentation by class I and class II molecules raises several crucial questions. (a) Why are the peptides derived from endocytosed antigens met only by class II but not class I molecules? (b) Why are class II molecules not always occupied and blocked by endogenously derived peptides, which they must encounter during their biosynthesis and transport from the ER to the endosomes?

The contrast to class I, the class II molecules are non-covalently associated with a third chain of 31 kDa, the non-polymorphic invariant chain (Ii; [11]) which, in gene-

[I 9225]

* This work was supported in part by grant DFG Hä 731/9-1.

▼ Present address: Instituto Nacional de la Nutricion "Salvador Zubiran", Tlalpan, Mexico.

Correspondence: Günter J. Hämmerling, German Cancer Research Center, Institute for Immunology and Genetics, Im Neuenheimer Feld 280, D-6900 Heidelberg, FRG

Abbreviations: **HEL:** Hen egg-white lysozyme **Ii:** Class II-associated invariant chain **ER:** Endoplasmic reticulum **BFA:** Brefeldin A

0014-2980/91/0505-1255$3.50+.25/0

ral, is coexpressed with class II antigens [12]. Ii associates with the class II α and β chains immediately after biosynthesis in the ER. This complex is transported through the Golgi compartment to the endosomes [13–16]. There, Ii is removed from the class II molecules and partially degraded into fragments of 21 kDa and 23 kDa [14]. Ii can also be found at the cell surface but is no longer associated with class II [17, 18]. The function of Ii is not known, but the features mentioned above have led to the speculation that Ii is involved in antigen processing and presentation by class II. Recently, Stockinger et al. [19] reported that I-E^d-transfected CA36.2.1 L cells [20] were much less efficient in the presentation of the C5 antigen to a T hybridoma than bulk cultures of CA36.2.1 cells supertransfected with Ii. However, these findings were somewhat obscured by the later observation that L cells can sometimes express their endogenous Ii chain. In addition, in another study employing I-A^d- and Ii-transfected L cells no influence of Ii on the presentation of OVA could be observed [21].

In the present study we investigated the contribution of Ii to antigen presentation in a different cell type and for different antigens. The antigens were hen egg-white lysozyme (HEL) and RNase, and as APC I-A^k- and Ii-transfected rat-2 fibroblasts were used, because rat-2 cells do not express their endogenous Ii. The results show clearly that Ii^+ transfectants process and present HEL more effectively than Ii^- cells. However, with the same set of transfectants no influence of Ii on the presentation of RNase was observed.

2 Materials and methods

2.1 Cell lines and T cell hybridomas

The thymidine kinase-defective rat fibroblast line rat-2 [22] was used as the recipient for transfections because it is Ii^-. As a professional APC the B cell hybridoma LK35.2 was used which is derived from a fusion of the A20B lymphoma with B10.BR-derived splenocytes [23]. It expresses the Ia alleles of both parents, I-A^k, I-A^d, I-E^k and I-E^d. The HEL- and RNase-specific T cell hybridomas are listed in Table 1. The fine specificity of the T hybridomas was deduced from the reactivity with synthetic HEL peptides. In the case of T hybridoma E.907 the respective peptide has not yet been identified, but it must be derived from the first 80 amino acids of HEL because cell transfected with a truncated HEL construct will stimulate this T hybridoma (Moreno, unpublished).

2.2 Genes

The cDNA for A^k_α (pKAK) and A^k_β (pKBK) were generously provided by D. Mathis, Strasbourg, France [29]. In these plasmids the promoters were exchanged by A. Heuser, Heidelberg, for the H-$2K^b$ gene-derived class I promoter, and the C_μ H chain enhancer was also introduced. The resulting constructs, pCMA (A^k_α) and pCMA (A^k_β), were a generous gift of A. Heuser, Heidelberg (unpublished). The mouse Ii gene was originally derived from the cosmid clone H-2^k 10.7 [30] and subcloned into the EMBL-8 vector which contains the neomycine resistance gene. The resulting construct p1Ii was donated by U. Pessara, Heidelberg [31]. The pkS5 plasmid contains also the cosmid 10.7 derived from the mouse Ii gene, but cloned into pUC19 (provided by K. Schenck, Heidelberg, unpublished). The neomycine resistance gene pAG60 was described by Brady et al. [32]. The plasmid containing the thymidine kinase gene pATK was constructed by and obtained from W. Pülm, Heidelberg (unpublished).

2.3 Antibodies and reagents

The rat In-1 mAb against mouse Ii [33], the anti-I-A^k antibodies H116-32 (α chain) and K22-203 (β chain) [34] and a rabbit serum against mouse Ii [35] have been used. HEL and RNase were purchased from Sigma (Munich, FRG).

2.4 Transfections

Rat-2 cells were transfected as described [30] following the calcium phosphate procedure of Wigler et al. [36]. For co-transfections, 10^6 adherent rat-2 cells in a 10-cm petridish were transfected with 10 μg pCMA (A^k_α) + 10 μg pCMB (A^k_β) + 1 μg p1Ii (Ii-neo). Selection of neomycine-resistant colonies was done in 0.3–0.6 mg/ml G418 (Gibco, Grand Island, NY). The resulting transfectants were designed RKKI (for rat-2, A^k_α, A^k_β, Ii). For generation of rat-2 cells expressing only I-A^k the cells were transfected with pCMA, pCMB + 1 μg pAG60 (neo^R). These transfectants were designated RKK. For supertransfection the RKK cells were transfected with the Ii plasmic pKS5 plus the thymidine kinase plasmid pATK. Selection was done in HAT medium. The transfectants were screened for I-A^k expression with antibodies H116-32 and K22-203 using a FACScan (Becton Dickinson, Heidelberg, FRG), and for Ii expression by immunoprecipitation.

2.5 Immunoprecipitation

The immunoprecipitation of Ii was done as described [30]. Briefly, 5×10^6 cells were labeled for 15 min with 50 μCi (= 1.85 MBq) [^{35}S]methionine. The cells were washed and lysed at 4 °C in 200 μl cold TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 1% NP40 plus protease inhibitors Trasylol (1/1000 dilution) and 10 mM PMSF. The debris were removed by centrifugation. For immunoprecipitation 20 μl In-1 hybridoma SN (20 × concentrated) and 30 μl of the protein A-binding mouse anti-rat κ chain antibody MAR 18.5 were added, followed by 10 μl protein A-Sepharose (50% suspension). After rotation overnight at 4 °C the precipitate was washed in TBS and separated by SDS-PAGE on a 15% gel. Gels were treated with PPO/DMSO for fluorography before drying, and then exposed to X-ray film at −70 °C.

2.6 Antigen presentation assay

The assay was done as described [26]. Briefly, T cell hybridomas (1×10^5/well) were incubated in triplicate in 96-well flat-bottom plates (Costar, Cambridge, MA)

together with 2.5×10^4 APC/well plus increasing amounts of antigen in a total volume of 200 µl. After 24 h at 37 °C 100 µl of SN was removed and cultured with 10 IL 2-dependent CTLL-2 cells for the determination of IL 2. After 20 h the cultures were probed with 1 µCi of [^{3}H]dThd (Amersham-Buchler, Braunschweig, FRG). Plates were incubated for additional 8 to 10 h prior to harvesting and counting in a liquid scintillation counter. HEL (molecular weight 14388) and bovine RNase A (molecular weight 13683) were purchased from Sigma.

2.7 Inhibition of antigen presentation by chloroquine and brefeldin A (BFA)

Inhibition of antigen presentation was performed as described previously [37]. Briefly, the APC were distributed into 96-well flat-bottom microtiter plates and preincubated with either 200 µM chloroquine or BFA (usually 20 µM, obtained from Sandoz AG., Basel, Switzerland) for either 30 min or 15 min, respectively, at 37 °C. After addition of antigen the plates were incubated for 4 h at 37 °C and then washed three times with RPMI 1640. After each centrifugation step (5 min, 1500 rpm) the SN were carfully removed with a 12-channel pipette. Next, the APC were fixed for 30 s in 100 µl of 0.05% glutaraldehyde. The fixation was stopped with 100 µM glycylglycine and the plates were washed again three times. The T hybridoma cells were then added and the assay was continued as described in the Sect. 2.6.

3 Results

3.1 T cell hybridomas and the generation of antigen-presenting rat-2 transfectants

The experimental approach was to compare antigen presentation by rat-2 cells transfected with class II alone or together with Ii. To minimize the possibility of intrinsic clonal variation among rat-2 clones with regard to their antigen presenting capacity, a panel of independent transfectants was produced and analyzed with a panel of T cell hybridomas directed against different HEL peptides. The hybridomas and their fine specificity are listed in Table 1, SEct. 2.1. This list also contains information about the relative sensitivity of the T hybridomas which was estimated in the present study using the LK35.2 B hybridoma as standard APC and incubation with increasing amounts of antigens. It can be seen that the most sensitive T hybridoma, 3A9, reaches 50% of its maximum response with as little as 0.1 µg/ml HEL, whereas other T hybridomas needed up to 300 times more HEL, *e.g.* T hybridoma 2C8.4.

For the generation of APC with defined expression of class II and Ii, rat-2 cells were transfected either with A^k_α and A^k_β alone, or with A^k_α, A^k_β plus the mouse Ii gene. The respective transfectants were designated RKK (for rat-2, A^k_α, A^k_β, followed by the clone number of the respective transfectants). RKK-poly indicates that all colonies from a transfection in a petri dish were mixed (about 100 colonies) and enriched for class II expression by cell sorting. The designation RKKI indicates that the rat-2 cells had been co-transfected with Ii. In the case of RKKI-poly not all cells are expected to express Ii. The relative amount of I-A^k on the surface of the transfectants was determined by FCM analysis with anti-I-A^k antibodies. Care was taken to select for further use transfectants expressing similar amounts of I-A^k. The presence and relative amount of Ii was determined by immunoprecipitation with the In-1 mAb [31]. Fig. 1a shows a typical example. Besides the characteristic band of M_r 31 kDa a minor band of 41 kDa can be seen which is the result of differential splicing of the Ii gene [29], and smaller bands of about 23 kDa which are degradation products. Some transfectants express relatively large amounts of Ii, for example, RKKI-16 and RKKI-53, whereas others express only low amounts of Ii, *e.g.* RKKI transfectants no. 1, 2, 32 and 36. To ascertain that the RKK transfectants, which did not receive the Ii gene, were indeed negative for their endogenous Ii, immunoprecipitation was done with a rabbit anti-mouse Ii serum, S22, which also cross-reacts with rat Ii. Even after overexposure of the gels for 14 days no band or only a faint one at M_r 31 kDa could be seen (Fig. 1b). Since this band was also observed in controls it probably reflects a nonspecific contaminant. The rat-2 transfectants used in this study are listed in Table 2.

3.2 Antigen presentation by I-A^k and Ii-transfected rat-2 fibroblasts

For antigen presentation assays 2.5×10^4 APC and 10^5 T hybridoma cells were mixed with increasing amounts of HEL. The B hybridoma LK35.2 was always included as a positive control for T cell activation. To ensure that the HEL

Table 1. T cell hybridomas[a]

T Hybridoma	Strain	Specificity (peptide)	Sensitivity[b] (µg/ml antigen)	Reference
3A9	CBA	HEL (46–61)	0.1	[24]
A.2B2	CBA	HEL (46–61)	0.7	[25]
2B5.1	C3H	HEL (46–61)	7.0	c)
1A3.2	C3H	HEL (46–61)	15.0	c)
E907.K	B6C3F$_1$	HEL (1–80)[d]	3.0	[26]
2A11	C3H	HEL (12–129)	0.3	c)
1D5	C3H	HEL (112–129)	1.0	c)
2C8.4	C3H	HEL (112–129)	3.0	[27]
TS12	CBA	RNase (43–56)	20.0	[28]

a) All T hybridomas are derived from fusions with the AKR thymoma BW5714.
b) The relative sensitivity or responsiveness of the T hybridomas was estimated by titrating HEL or RNase on LK35.2B hybridoma cells as APC. Values represent the amount of antigen at which 50% of the maximal response was obtained.
c) L. Adorini, unpublished.
d) Exact peptide not known; it is derived from the first 80 amino acids of HEL.

did not contain degraded material it was purified by HPLC for some experiments. Fig. 2 shows the results obtained with the most sensitive T hybridoma, 3A9. It responds to RKKI cells expressing relatively large amounts of Ii (RKKI-16, 53 and poly) at fairly low concentrations of HEL, ranging between 0.5 to 10 μg/ml (Fig. 2a). RKKI-53,

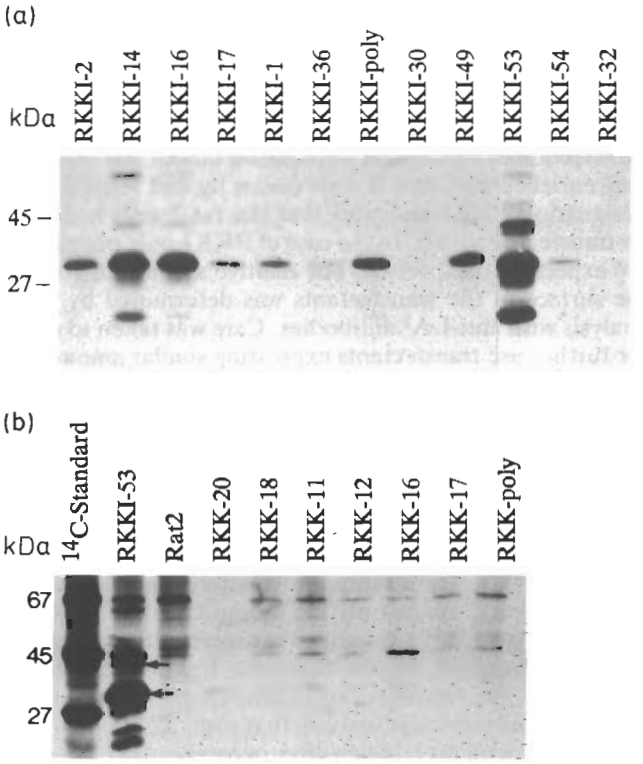

Figure 1. Expression of Ii in rat-2 transfectants. (a) The Ii chain in RKKI transfectants was immunoprecipitated with the In-1 mAb. Exposure time of the autoradiograph: 68 h. (b) Rat-2 cells or RKK transfectants were immunoprecipitated with the rabbit anti-mouse Ii serum S22 which also cross-reacts with rat Ii. The Ii^+ transfectant RKKI-43 was included as a positive control. Exposure time of the autoradiograph: 14 days.

Table 2. Expression of I-A^k and Ii in transfected Rat-2 fibroblasts[a)]

Group	Cell line	Expression of I-A^k[b)]	Expression of Ii
I	Rat-2	6	–
	RKKI-16	95	++
	RKKI-33	92	++
	RKKI-53	92	++
	RKKI-poly	102	++
II	RKKI- 1	84	+
	RKKI- 2	93	+
	RKKI-32	100	+/–
	RKKI-36	92	+/–
III	RKK-11	70	–
	RKK-12	89	–
	RKK-16	89	–
	RKK-17	104	–
	RKK-18	90	–
	RKK-20	112	–
	RKK-poly	115	–

a) Rat-2 cells were transfected with A^k_α and A^k_β cDNA plus the Ii gene (designated RKKI). Transfectants from group 3 were not co-transfected with Ii (designated RKK). The expression of I-A^k was determined with a FACScan. The expression of Ii was estimated by immunoprecipitation. ++ Indicates relatively strong expression, + indicates intermediate or weak, and – indicates no expression, as judged from Fig. 1a and b. For comparison, LK35.2 cells would score as +++.

b) Relative fluorescence channel.

which has the highest level of Ii, was in all tests the best APC. Transfectants expressing only low amounts of Ii (RKKI-1, 2, 3, 32 and 36) require 50 to 200 μg/ml HEL for a similar 50% response (Fig. 2b), whereas most transfectants without Ii require even higher HEL concentrations (Fig. 2c). Thus, it appears that there is a correlation between the amounts of Ii and the ability of the transfectants to process and present HEL to 3A9. However, it is also apparent that the LK35.2 cells are more potent APC

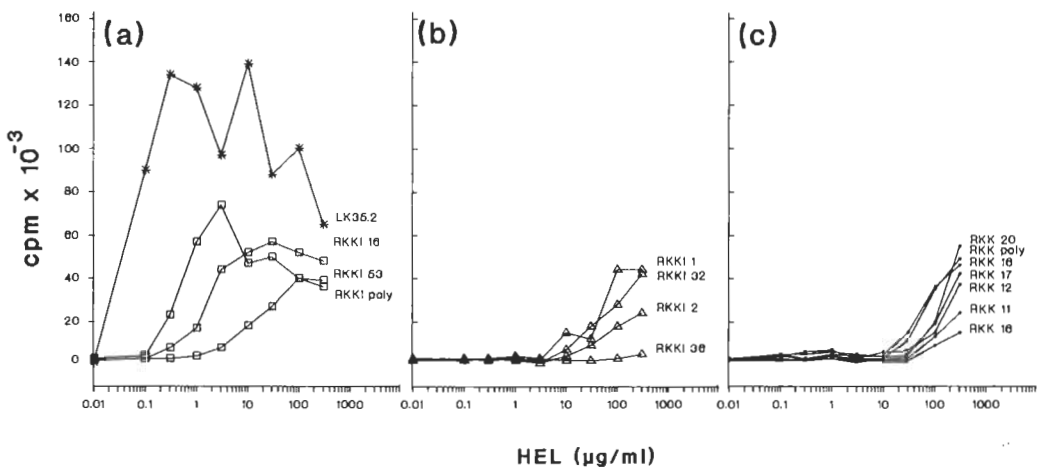

Figure 2. Ii augments antigen presentation of HEL. Rat-2 transfectants were incubated with increasing amounts of HEL plus the HEL-specific T hybridoma 3A9. LK35.2 cells were included as a positive control. (a) shows transfectants expressing relatively large amounts of Ii (squares), (b) shows transfectants expressing only low amounts of Ii (triangles), transfectants in (c) are Ii^- (circles) (see Table 2).

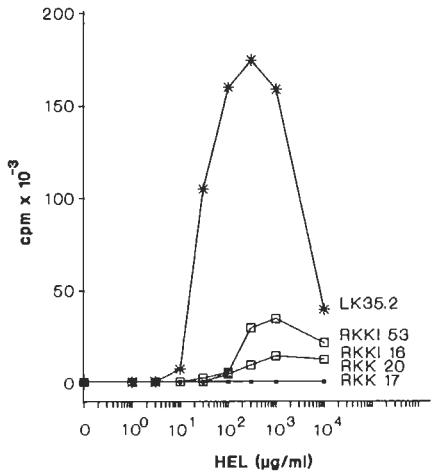

Figure 3. Ii augments the presentation of HEL to the T hybridoma 1A3.2 requiring high HEL concentration for optimal stimulation. The Ii$^+$ RKKI-53 and RKKI-16 (squares), and the Ii$^-$ transfectants RKK-20 and RKK-17 (circles) were incubated with increasing amounts of HEL plus the T hybridoma 1A3.2.

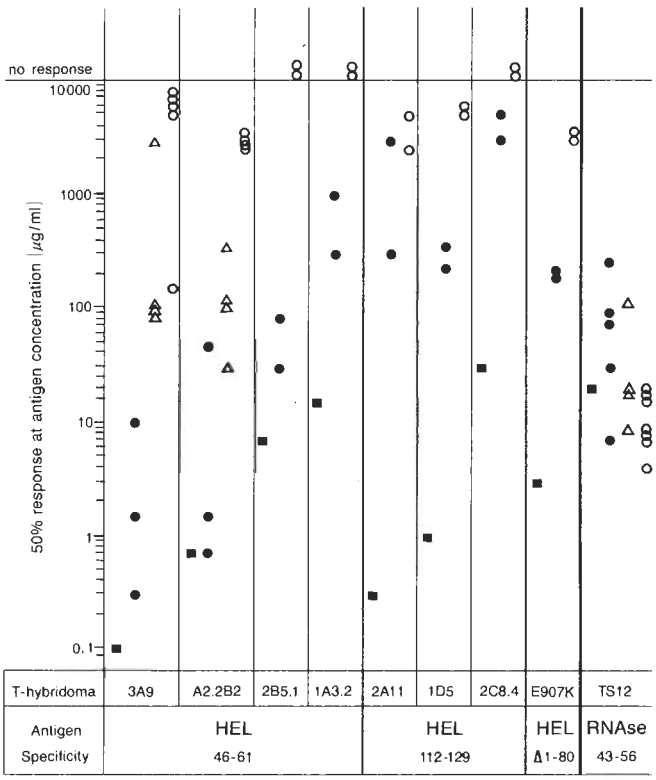

Figure 4. Summary of results: Ii increases the ability of rat-2 transfectants to present HEL to T hybridomas with specificity for different HEL peptides but not for the presentation of RNase. The 50% values represent the concentration of antigen (µg/ml) at which 50% of the maximum response was obtained. These values have been estimated from the titration curves which, for example, are shown in Figs. 2 and 3. At higher concentrations the respective values represent only rough estimates. The following APC were used: LK35.2 (■); RKKI transfectants expressing relatively large amounts of Ii (●); RKKI transfectants expressing low amounts of Ii (△); Ii$^-$ RKK transfectants (○). The figure contains also the corresponding data obtained with the RNase specific T hybridoma TS12 (see Fig. 7).

than the best transfectants. Possibly, LK35.2 processes antigens more efficiently. In addition, the LK35.2 cells seem to express Ii and I-A^k at higher levels than the transfectants.

These observations were verified with other I-A^k-restricted T hybridomas with specificity for the HEL peptide 46–61. The strongly Ii$^+$ transfectants were always more efficient APC, regardless of the sensitivity of the T hybridomas. Fig. 3 shows the data for the relatively insensitive T hybridoma 1A3.2, RKKI-53 and RKKI-16 are able to generate a weak response, whereas the Ii$^-$ transfectants fail to do so.

It was of importance to see whether or not Ii would influence the presentation of only particular peptides derived from HEL. Therefore, additional T hybridomas were employed which were specific for peptides 112–129 (T hybrids 2A11, 1D5 and 2C8.4), or for the unidentified peptide (× 1–80) derived from the first 80 amino acids of HEL (T hybrid E907.K, see Sect. 2.1). As APC the transfectants RKKI-53, RKKI-16, RKK-17 and RKK-20 were chosen. The respective data are compiled as a summary in Fig. 4 which displays the concentration of HEL at which a 50% maximum response was obtained. At higher concentrations of HEL (10 mg/ml) the values represent sometimes only a rough extrapolation because high concentrations of HEL can be inhibitory (see for example the curve for LK35.2 in Figs. 2a and 3). In some cases no response with Ii$^-$ transfectants was obtained at 10.mg/ml HEL, as indicated on the top of Fig. 4. Although there is considerable variation in the sensitivity and responsiveness of the T hybridomas the data show that RKKI transfectants with large amounts of Ii (black circles) are always more potent APC than RKK without Ii (open circles). This result is true for all three HEL peptide-specific hybridomas recognizing the HEL peptides 46–61, 112–129 and (× 1–80) defined by E907.K, but the effect appeared to be more pronounced for peptides 46–61 than for 112–129 and (× 1–80). When RKKI transfectants expressing low amounts of Ii were tested, they were found to be low or intermediate antigen presenters (triangles in Fig. 4).

There may be some clonal variation with regard to the antigen presentation capacity of the transfectants which is independent of Ii. Therefore, several RKK clones were supertransfected with Ii. In no case could a high level of Ii expression be achieved, possibly because for the super-

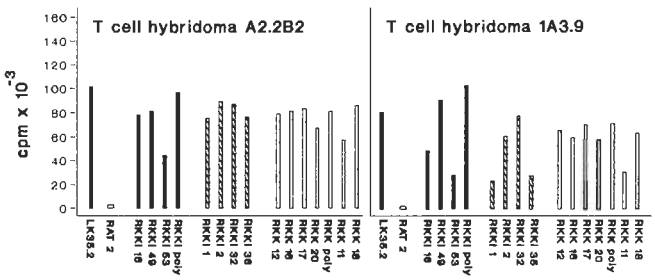

Figure 5. Presentation of the HEL peptide 46–61 by Ii$^+$ and Ii$^-$ transfectants. The Ii$^+$ and Ii$^-$ rat-2 transfectants were incubated with the HEL peptide 46–61 (10 µM) plus the T hybridomas 3A9 or A2B2.2.

transfectants a different Ii vector was used from that used to obtain the RKKI cotransfectants. The Ii supertransfectants displayed also improved presentation of HEL to the T hybridoma 3A9 (factor of 3 to 10), but the augmentation was not as drastic as that seen with the primary RKKI transfectants, such as RKKI-53, probably due to the relatively low level of Ii (data not shown).

3.3 Presentation of HEL peptides

We also investigated whether Ii would influence not only the presentation peptides derived from HEL processing but also the presentation of the synthetic HEL peptide 36–61 which does not require processing. It can be seen from Fig. 5 that presentation of the peptide by the Ii^+ and Ii^- rat-2 transfectants resulted in a similar stimulation of the T hybridomas A2.2B2 and 3A9. Titrations of the minimal-sized stimulatory peptide HEL 52–61 did not reveal differences between RKKI-53 and RKK-17 cells (Fig. 6). The data presented in Fig. 6 also underline that the T hybridoma 2B5.1 is less sensitive than 3A9.

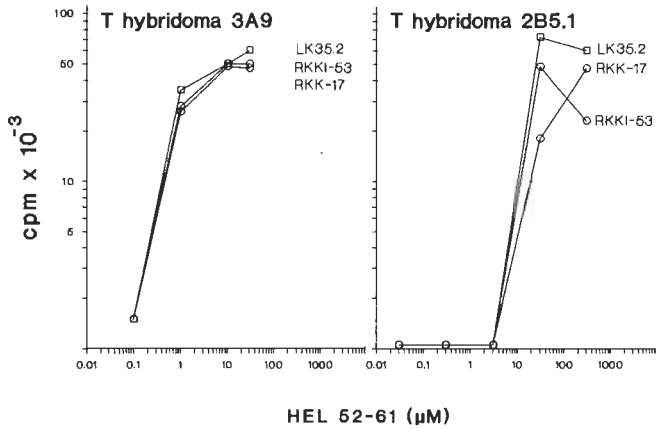

Figure 6. Presentation of the HEL peptide 52–61. Increasing amounts of the synthetic HEL peptide 52–61 were incubated with the Ii^+ transfectant RKKI-53 (○) and the Ii^- transfectant RKK-17 (●) plus the highly sensitive T hybridoma 3A9 (a) or the relatively insensitive T hybridoma 2B5.1 (b). LK35.2 APC were included as positive control (squares).

3.4 Presentation of the antigen RNase is not augmented by Ii

Using the same set of transfectants we studied whether the presentation of another antigen, RNase, was also augmented by Ii. In contrast to the findings with HEL the results presented in Fig. 7 demonstrate that all transfectants present RNase approximately to the same extent regardless of their Ii expression. These data are also summarized in Fig. 4.

3.5 Quantitative differences in the inhibition of presentation of RNase and HEL by BFA

Adorini et al. [37] have reported that the presentation of HEL requires *de novo* synthesized class II molecules as demonstrated by inhibition with the drug BFA which blocks the egress of newly synthesized proteins from the ER into the Golgi compartment [38, 39]. Because of the observed differences in the requirement of Ii for the presentation of HEL and RNase, we tested whether the presentation of RNase would also require newly synthesized class II, since one could speculate that some antigens are presented by a pool of pre-existing class II, such as recycled class II molecules which have been described [40–42].

Interestingly, only the presentation of HEL was inhibitable by BFA at all HEL concentrations tested. In striking contrast, a significant inhibition by BFA of RNase presentation was observed at low concentrations of RNase (< 3 μM) whereas at higher concentrations no inhibition was found with both RKKI-53 and RKK-17 as APC (Fig. 8). In confirmation of previous studies [27, 37] the presentation of both antigens could be blocked efficiently by chloroquine suggesting that both antigens require a low pH compartment for processing. With RKK-17 the inhibi-

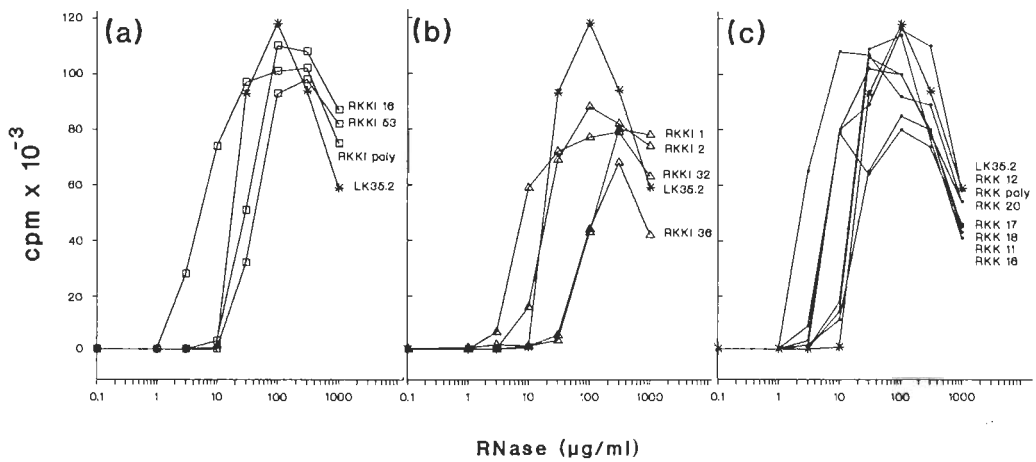

Figure 7. Presentation of the antigen RNase is not augmented by Ii. The capacity of Ii^+ and Ii^- transfectants to present the antigen RNase to the T hybridomas TS12 was assessed by incubation with increasing amounts of RNase. (a) Shows the results obtained with the RKKI transfectants expressing relatively large amounts of Ii (□), (b) shows transfectants expressing only low amounts of Ii (△), (c) shows RKK transfectants expressing no Ii (○) LK35.2 (*). These 50% response values are tabulated in Fig. 4.

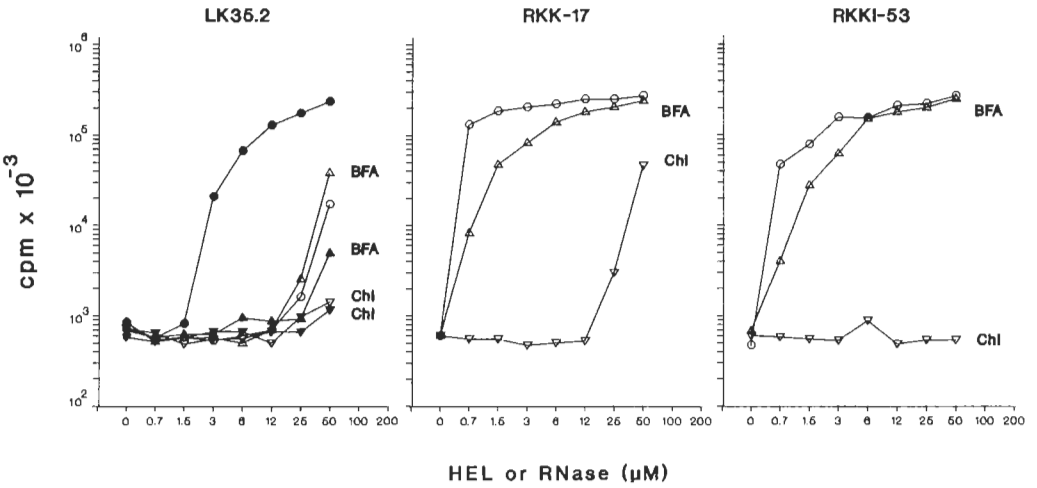

Figure 8. Presentation of RNase is not blocked by brefeldin A. The APC were pulsed with HEL or RNase in the presence of 20 μM BFA (△) or 200 μM chloroquine (▽) or no inhibitor (○). After fixation the T hybridomas were added and the Ii2 production was determined. Closed symbols: presentation of HEL measured with T hybridoma 2C8.4. Open symbols: presentation of RNase measured with T hybridoma TS12.

tion by chloroquine was incomplete at high concentrations of RNase. The reasons for this are not clear.

4 Discussion

The most striking result of the present study is that the Ii augments the antigen-presenting capacity of class II-transfected rat-2 fibroblasts for the antigen HEL but not for RNase (see Fig. 4 for a summary of results). In the case of HEL the data suggest the existence of a quantitative relationship between the amount of Ii and the antigen-presenting capacity. Transfectants expressing large amounts of Ii were, in general, superior to transfectants expressing only low amounts. The use of a panel of independent transfectants and supertransfectants showed that the correlation between expression of Ii and improved efficiency of antigen presentation was not fortuitous or due to clonal variation. That such clonal variation can exist is, for example, suggested by the Ii^- transfectant RKK-20 which, in most assays, was more efficient than the other Ii^- cells. The effect of Ii was not peptide specific because it was observed for T cell hybridomas specific for the HEL peptides 46–61, 112–129 and an as yet unidentified peptide (× 1–80), derived from the first 80 amino acids of HEL. In addition, the positive effect of Ii was also apparent in the case of relatively insensitive T hybridomas requiring large amounts of antigen for optimal stimulation, such as 1A3.2.

Presentation of synthetic peptides was performed by Ii^+ and Ii^- transfectants with similar efficiency (Figs. 5 and 6). Peterson and Miller [21] have reported that Ii has an inhibitory effect on peptide presentation. The reasons for this discrepancy are not clear but they may be related to a different level of Ii expression in the respective transfectants.

Altogether the data suggest that Ii is involved in intracellular events during antigen presentation, such as processing of HEL and/or association of the resulting HEL peptides with class II molecules. Several hypotheses have been suggested to explain the role of Ii in antigen presentation. First, it has been proposed that in the ER the Ii chain covers the peptide binding groove of class II and thereby prevents it from being occupied by endogenously derived peptides [43, 44]. After the transport to the endosomal compartment the Ii chain is removed and the binding site of class II molecules is made available for peptides derived from endocytosed antigens [14]. The fact that the Ii/class II complex is transported from the ER to the endosomes fits with this suggestion. Moreover, Roche and Cresswell [45] recently presented biochemical evidence that isolated DR5 α/β dimers bound radiolabeled influenza hemagglutinin peptide more efficiently than DR5 complexed with Ii, suggesting that Ii blocks the binding of peptides to class II. Similar data were recently also reported by Teyton et al. [46].

Another hypothesis to explain the role of Ii is the "sorting hypothesis" [44] which addresses the question of why endocytosed antigen is met in the endosomes predominantly by class II but not by class I molecules. The Ii chain may contain a sorting signal which targets the Ii/class II complex from the Golgi compartment into the endosomal compartment. This would explain why mainly class II but not class I is found in the endosomes. Indeed, Bakke and Dobberstein [47] have observed that after removal of the first 15 amino acids from the N-terminus, the Ii chain was not anymore transported to the endosomes. Obviously, this hypothesis is not in conflict with the observation that Ii influences the binding of peptides by class II. Thus, the picture emerges that Ii seems to have at least two functions: sorting and blocking.

However, these hypotheses do not explain why Ii appears to be important only for some but not all antigens. Using the same set of transfectants as APC the present study demonstrates that only the presentation of HEL but not that of RNase is augmented by Ii (see Figs. 4 and 7). Since only one T hybridoma with specificity for RNase (peptide 43–56) was available we cannot role out the formal

possibility that only the presentation of this peptide but not of other RNase-derived peptides was independent of Ii, but the differential inhibition of HEL and RNase presentation by BFA (Fig. 8) indicates that there are differences in the presentation of these two antigens. To our knowledge, BFA-mediated inhibition of antigen presentation by class II has been observed for the exogenous antigens HEL, pigeon cytochrome *c* [37] and OVA [48], suggesting that for these antigens *de novo* synthesis of class II is required. RNase seems to be the first exception, but more antigens have to be investigated. However, the lack of inhibition of RNase presentation by BFA is not absolute, as at lower antigen concentrations inhibition was found (Fig. 8). Our observations suggest that the presentation of low concentrations of RNase is (at least partially) mediated by *de novo* synthesized class II molecules, whereas at higher concentrations RNase interacts with a pool of pre-existing class II molecules. This assumption would also imply that due to their different molecular characteristics HEL and RNase are endocytosed into distinct intracellular compartments, were they could meet the different pools of class II. Since RNase is known to be a very stable and relatively acid-resistant protein it is possible that it penetrates deeper into the endosomal/lysosomal compartment than HEL. Quantitative differences in the degradation and intracellular location of various endocytosed proteins have been reported [49]. In this context it is of interest to note that RNase but not HEL carries a KFERQ motif which in starving or stressed cells facilitates transfer to and enhanced degradation in lysosomes. This motif binds also to a heat shock protein, the prp73 peptide recognition protein, which can also bind to clathrin [50]. These differences between HEL and RNase are intriguing, but whether they direct HEL and RNase into different intracellular compartments which are involved in antigen processing is not yet clear. Nothing is known about these hypothetical distinct compartments, but obviously all of them require low pH for antigen processing.

According to this hypothesis only the pool of class II which is required for presentation of HEL would be associated with Ii, but not the pre-existing pool required for presentation of RNase. This pool of Ii^- class II molecules could be derived from the class II/Ii complex after removal of Ii in an endosomal compartment, or it could represent class II which had failed to associate with Ii in the ER and which, therefore, may have taken a (partially) different intracellular pathway. It is also possible that the endocytosed RNase is met by recycled class II molecules which could be derived from any of the class II pools. Our results do not answer this latter question. Recently, it has been reported that B cells but not fibroblasts recycle their class II molecules [51]. If recycled class II molecules were indeed involved in the presentation of RNase one would have expected that the LK35.2 cells present RNase much better than the transfected fibroblasts, which in our experiments is not the case. In fact, in some experiments the fibroblasts presented RNase more efficiently than B cells (*e.g.* Fig. 8), although the latter express more class II molecules. This question needs to be investigated in more detail.

It should be noted that there exist conflicting reports with regard to the enhancement of antigen presentation by Ii. Augmentation by Ii was found for C5, influenza virus [19], and HEL (present study), but not for OVA [21], cytochrome *c* (R. Germain, personal communication) and RNase (present study). However, there does not seem to be a general correlation between the independence of antigen presentation from Ii and the lack of inhibition by BFA, because the presentation of OVA could be blocked by BFA [37, 48]. Altogether, the observations presented here strongly suggest that there exist distinct mechanisms or pathways for the processing and presentation of different protein antigens and that not all pathways depend on Ii. It is anticipated that the relative contribution of the various mechanisms to the overall immune response to protein antigens can be elucidated in Ii^- mice. Further studies are in progress.

We thank P. Allen, J. McCluskey, D., Mathis and C. Benoist for the T hybridomas 3A9, A2.2B2 and TS12. We appreciate the expert technical help of N. Bulbuc and M. Post, and thank B. Ermshaus for the skillful preparation of the manuscript.

Received January 16, 1991.

5 References

1 Townsend, A., Gotch, F. M. and Davey, J., *Cell* 1985. *42:* 457.
2 Allen, P. M., Babbit, B. P. and Unanue, E. R., *Immunol. Rev.* 1987. *98:* 171.
3 Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennet, W. S., Strominger, J. L. and Wiley, D. C., *Nature* 1987. *329:* 506.
4 Moore, M. M., Carbone, F. R. and Bevan, M. J., *Cell* 1988. *54:* 777.
5 Ziegler, H. K. and Unanue, E. R., *Proc. Natl. Acad. Sci. USA* 1982. *79:* 175.
6 Jin, Y., Shih, J.W.-K. and Berkower, I., *J. Exp. Med.* 1988. *168:* 293.
7 Jacobson, S., Sekaly, R. P., Jacobson, C. L., McFarland, H. F. and Long, E. O., *J. Virol.* 1989. *63:* 1756.
8 Eisenlohr, L. C. and Hackett, C. J., *J. Exp. Med.* 1989. *169:* 921.
9 Nuchtern, J. G., Biddison, W. E. and Klausner, R. D., *Nature* 1990. *343:* 74.
10 Thomas, D. B., Hodgson, J., Riska, P. F. and Graham, C. M., *J. Immunol.* 1990. *144:* 2789.
11 Jones, P. P., Murphy, D. B., Hewgill, D. and McDevitt, H. O., *Immunochemistry* 1978. *16:* 51.
12 Momburg, F., Koch, N., Möller, P., Moldenhauer, G., Butcher, G. W. and Hämmerling, G. J., *J. Immunol.* 1986. *136:* 940.
13 Cresswell, P., *Proc. Natl. Acad. Sci. USA* 1986. *82:* 8188.
14 Blum, J. S. and Cresswell, P., *Proc. Natl. Acad. Sci. USA* 1988. *85:* 3975.
15 Guagliardi, L. E., Koppelman, B., Blum, J. S., Marks, M. S., Cresswell, P. and Brodsky, F. M., *Nature* 1990. *343:* 133.
16 Neefjes, J. J., Stollorz, V., Peters, P. J., Geuze, H. J. and Ploegh, H. L., *Cell* 1990. *61:* 71.
17 Koch, N., Koch, S. and Hämmerling, G. J., *Nature* 1982. *299:* 644.
18 Wraight, C. J., van Endert, P., Möller, P., Lipp, J., Ling, N. R., MacLennan, I. C. M., Koch, N. and Moldenhauer, G., *J. Biol. Chem.* 1990. *265:* 5787.
19 Stockinger, B., Pessara, U., Lin, R. H., Habicht, J., Grez, M. and Koch, N., *Cell* 1989. *56:* 683.
20 Shastri, N., Malissen, B. and Hood, L., *Proc. Natl. Acad. Sci. USA* 1985. *82:* 5885.
21 Peterson, M. and Miller, J., *Nature* 1990. *345:* 172.
22 Topp, W. C., *Virology* 1981. *113:* 408.
23 Kappler, J., White, J., Wegmann, D., Mustain, E. and Marrack, P., *Proc. Natl. Acad. Sci. USA* 1982. *79:* 3604.

24 Allen, P. M. and Unanue, E. R., *J. Immunol.* 1984. *132:* 1077.
25 Allen, P. M., McKean, D. J., Beck, B. N., Sheffield, J. and Glimcher, L., *J. Exp. Med.* 1985. *162:* 1264.
26 Moreno, J. and Lipsky, P. E., *J. Immunol.* 1986. *136:* 3579.
27 Adorini, L., Appella, E., Doria, G. and Nagy, Z., *J. Exp. Med.* 1988. *168:* 2091.
28 Lorenz, R. G., Tyler, A. N. and Allen, P. M., *J. Immunol.* 1988. *141:* 4124.
29 Landais, D., Beck, B. N., Buerstedde, J.-M., Degraw, D., Klein, D., Koch, N., Murphy, D., Pierres, M., Tada, T., Yamamoto, K., Benoist, C. and Mathis, D., *J. Immunol.* 1986. *137:* 3002.
30 Yamamoto, K., Koch, N., Steinmetz, M. and Hämmerling, G. J., *J. Immunol.* 1985. *134:* 3461.
31 Pessara, U., Momburg, F. and Koch, N., *Eur. J. Immunol.* 1988. *18:* 1719.
32 Brady, G., Jantzen, H. M., Bernard, H. U., Brown, R., Schütz, G. and Hashimoto-Gotoh, T., *Gene* 1984. *27:* 233.
33 Koch, N., Koch, S. and Hämmerling, G. J., *Nature* 1982. *299:* 644.
34 Koch, N., Hämmerling, G. J., Tada, N., Kimura, S. and Hämmerling, U., *Eur. J. Immunol.* 1982. *12:* 909.
35 Koch, S., Schultz, A. and Koch, N., *J. Immunol. Methods* 1987. *103:* 211.
36 Lorenz, R. G., Tyler, A. N. and Allen, P. M., *J. Immunol.* 1988. *141:* 4124.
37 Adorini, L., Ullrich, J. S., Appella, E. and Fuchs, S., *Nature* 1990. *346:* 63.
38 Nuchtern, J. G., Bonifacino, J. S., Biddison, W. E. and Klausner, R. D., *Nature* 1989. *339:* 223.
39 Yewdell, J. W. and Bennink, J. R., *Science* 1989. *244:* 1072.
40 Pernis, B., *Immunol. Today* 1985. *6:* 45.
41 Harding, C. V. and Unanue, E. R., *J. Immunol.* 1989. *142:* 12.
42 Reid, P. A. and Watts, C., *Nature* 1990. *346:* 655.
43 Elliott, W. L., Stille, C. J., Thomas, L. J. and Humphreys, R. E., *J. Immunol.* 1987. *138:* 2949.
44 Long, E. O., *Immunol. Today* 1989. *10:* 232.
45 Roche, P. A. and Cresswell, P., *Nature* 1990. *345:* 615.
46 Teyton, L., O'Sullivan, D., Dickson, P. W., Lotteau, V., Sette, A., Fink, P. and Peterson, P. A., *Nature* 1990. *348:* 39.
47 Bakke, O. and Dobberstein, B., *Cell* 1990. *63:* 707.
48 Pierre, Y. S. and Watts, T. H., *J. Immunol.* 1990. *135:* 812.
49 Tassin, M.-T., Lang, T., Antoine, J.-C., Hellio, R. and Ryter, A., *Eur. J. Cell Biol.* 1990. *52:* 219.
50 Dice, J. F., *Trends Biochem. Sci.* 1990. *15:* 305.
51 Saamero, J., Humbert, M., Cosson, P. and Davoust, J., *EMBO J.* 1990. *9:* 3489.