A vast portion of the immunological literature has been dedicated, in the last 15 years, to the role of the major histocompatibility complex (MHC) in the immune response. There is now agreement (1, 2) that the molecules coded by the genes of the MHC are essential for cellular interactions and ultimately for self, not-self distinction, at least for what concerns the response of the T system and for the production of antibodies to T-dependent antigens.

In spite of the extensive efforts and of the considerable advances in the immunogenetics and biochemistry of the MHC system, several important aspects of the immune function of this system are still unknown.

We have chosen to re-examine the cytology of histocompatibility antigens, with a view to some more recently investigated aspects of cell receptor dynamics, as a contribution to the knowledge of the cellular basis of MHC function. This paper is dedicated to Class I MHC molecules and T cells. It shows that Class I MHC molecules are spontaneously internalized at a rapid rate by activated T lymphoid cells in a manner that has many similarities with the recently elucidated internalization and recycling of different receptors in nonimmunocytes (3).

Materials and Methods

**Mice.** Female BALB/c, A/J, and C3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, ME. C3H.OL mice were obtained from Dr. A. Augustin, Columbia University, New York and bred in our animal facilities.

**Cell Culture.** Single cell suspensions were obtained from spleens of unprimed mice 4–8 wk of age. Erythrocytes were removed by lysis with NH₄Cl buffer and unfraccionated splenocytes were cultured at 10⁶ cells/ml in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml Fungizone, 20 mM glutamine, and 5 × 10⁻⁵ M 2-mercaptoethanol in the absence or presence of either 2 µg/ml concanavalin A (Con A), or 20 µg/ml lipopolysaccharide (LPS), or as a 1:1 two-way mixed-lymphocyte reaction (MLR). All tissue culture reagents were purchased from Grand Island Biological Co.,
Grand Island, NY. Con A was purchased from Pharmacia Fine Chemicals, Piscataway, NJ and LPS (E. coli 055:B5) was obtained from Difco Laboratories, Detroit, MI. Viable cell counts were determined by trypan blue exclusion.

**Antibodies.** Hybridoma cell lines 20-8-4S (4) and 3-83P (5) were generously provided by Dr. D. Sachs, NIH, Bethesda, MD. Hybridoma H0-13-4 (6) was obtained from the Salk Institute. Monoclonal antibodies 20-8-4S (anti-K\(^a\), mouse IgG\(_2a\)) and 3-83P (anti-K\(^k\), D\(^k\) mouse IgG\(_2a\)) were purified from cell culture supernatant by affinity chromatography over Protein-A Sepharose. Monoclonal antibody H0-13-4 (anti-Thy-1.2, mouse IgM) was purified over a rabbit anti-mouse immunoglobulin affinity column. Ascites from 20-8-4S was also provided by Dr. D. Sachs. Monoclonal rat anti-mouse T200 antibody 30-G12 (7) was kindly supplied by Dr. W. Cleveland, Columbia University, New York as a hybridoma culture supernatant. Affinity-purified goat anti-Con A was the generous gift of Dr. P. Kaladas, Columbia University, New York. Rabbit anti-mouse immunoglobulin (Ig) and rabbit anti-goat Ig antibodies were purified from antisera as previously described (8). Goat anti-rat Ig was purchased from Cappel Laboratories, West Chester, PA.

**Immunofluorescence.** Purified antibodies were conjugated to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) by the celite method (9). Affinity-purified antibodies were also conjugated to biotin as described by Guesdon et al. (10). Diatomaceous earth, FITC, and TRITC were purchased from Sigma Chemical Co., St. Louis, MO. N-Hydroxysuccinimidobiotin was obtained from Pierce Chemical Co., Rockford, IL. FITC- and TRITC-avidin were purchased from Vector Laboratories, Inc., Burlingame, CA.

Cells harvested from culture were washed three times with chilled Hanks' Balanced Salt Solution (HBSS) containing 5% FCS and 10 mM Na azide. Membrane antigens were stained in suspension at 0°C. For detection of intracytoplasmic antigens, cytocentrifuge smears were prepared, fixed in 95% ethanol at -20°C for 15 min, and stained at room temperature. Preparations were viewed using a Leitz Dialux Microscope with vertical illumination and optical systems for the selective visualization of fluorescein or rhodamine. Staining methods are essentially as described elsewhere (8).

**Internalization of Anti-H-2K Antibodies.** BALB/c splenocytes cultured for 3 d in Con A were washed three times with HBSS containing 5% FCS and replated at 2 \(\times\) 10\(^6\) cells/ml in fresh medium at 37°C. FITC-anti-K\(^4\) was added to a final antibody concentration of 10 \(\mu\)g/ml. Cells were harvested at various times and washed with chilled HBSS containing 5% FCS and 10 mM Na azide. Control cells were prechilled for 1 h, then incubated in anti-K\(^4\) at 0°C. Cytocentrifuge smears were prepared, fixed in 95% ethanol, and stained with TRITC-anti-K\(^4\). Percent cells containing fluorescein- or rhodamine-positive vesicles were enumerated.

In addition, the internalization of anti-H-2K antibodies was studied in mouse spleen cells exposed for a prolonged period. Unstimulated mouse spleen cells or cells cultured for 54 h with Con A or LPS, as indicated above, were exposed to unconjugated 20-8-4S ascites at a final dilution of 1:5,000 for 18 h at 37°C. They were harvested, washed, and stained in suspension for membrane anti-K\(^4\) using TRITC-rabbit anti-mouse IgG\(_2a\). Cytocentrifuge smears were then prepared, fixed, and stained with FITC-rabbit anti-mouse IgG to localize internalized anti-K\(^4\) antibodies.

**Effect of Cycloheximide or Monensin on Membrane and Intracytoplasmic H-2K Antigen Expression.** BALB/c splenocytes were first cultured for 3 d in Con A. Cycloheximide (Calbiochem-Behring Corp., San Diego, CA) was dissolved in Dulbecco's phosphate-buffered saline and added to a final concentration of 50 \(\mu\)M. Cells were harvested at various times afterwards, washed, and stained in suspension using FITC-anti-K\(^4\). Surface fluorescence was measured on a FACS-IV cell sorter (Becton, Dickinson & Co., Mountain View, CA). 1 \(\times\) 10\(^4\) cells were analyzed for each sample. Cytocentrifuge smears were prepared from the same samples, fixed in ethanol, and stained with TRITC-anti-K\(^4\).

Monensin (Calbiochem-Behring Corp., La Jolla, CA) was dissolved in 95% ethanol to 5 mM and added to Con A-activated splenocytes to a final concentration of 25 \(\mu\)M. Cells were harvested and analyzed for surface and intracellular H-2K as described for cycloheximide-treated cells. An equivalent volume of ethanol was added to control cultures.
To determine the effect of cycloheximide or monensin on the internalization of anti-H-2K antibodies, Con A-activated splenocytes were pretreated with 50 μM monensin for 3 h. FITC-anti-K^d was then added to a final concentration of 10 μg/ml. Cells were harvested after 1 h and washed with chilled HBSS containing 5% FCS and 10 mM Na azide. Cytocentrifuge smears were prepared, fixed in 95% ethanol, and restained with TRITC-anti-K^d. Percent cells containing fluorescein- or rhodamine-positive vesicles were enumerated.

Results

Activated Murine T Cells Contain Intracytoplasmic Vesicles with Class I MHC Molecules. Immunofluorescence examination of fixed cells with fluorochrome-conjugated monoclonal antibodies specific for murine Class I MHC molecules showed the presence of numerous intracytoplasmic vesicles containing these molecules in activated T lymphoid cells (Table I). The intracytoplasmic location of these vesicles was confirmed by differential membrane and cytoplasmic staining (Fig. 1, a and b), performed as indicated in Materials and Methods; the specificity was assured by absence of staining of cells from mice of an H-2 haplotype different from that with which the anti-H-2K antibodies were reactive. Under conditions in which the pattern of intracytoplasmic vesicles with Class I MHC molecules was fully developed (such as in cultures of murine spleen cells stimulated for 3 d with Con A), a substantial proportion of the lymphoid cells contained these structures. Each positive cell contained 10–20 vesicles; the size of the vesicles was fairly uniform and corresponded to ~1/25th of the apparent diameter of the flattened cells (0.6–0.9 μm apparent vesicle diameter). The vesicles were distributed in the cytoplasm in a random fashion.

Associated membrane staining using biotinylated monoclonal anti-Thy-1.2 and biotinylated rabbit anti-mouse Ig with a conjugated-avidin sandwich identi-

### Table I

| Expt. | Mouse strain | H-2 Haplotype | Percent cells with intracellular H-2K |
|-------|--------------|---------------|-------------------------------------|
|       |              |               | d.0 Un-treated | d.3 Un-treated | d.3 Con A | d.3 MLR | d.3 LPS |
| 1     | BALB/c       | d             | 1.8           | 3.1           | 48.8      | 18.9*    | 1.0     |
| 2     |              |               | 1.6           | 9.4           | 71.6      | 15.4*    | 2.6     |
| 3     |              |               | 0             | 3.0           | 60.8      |          |         |
| 4     |              |               | 0             | 3.0           | 42.2      | 15.4*    | 2.6     |
| 5     | C3H.OL       | ol            | 2.8           | 2.7           | 23.0      |          |         |
| 6     |              |               | 0.9           | 4.0           | 35.8      |          |         |
| 7     |              |               | 0.8           | 9.0           | 33.8      | 15.7*    |         |
| 8     | C3H/HeJ      | k             | 0             | 16.7          | 58.2      | 36.1*    |         |
| 9     |              |               | 5.3           | 14.7          | 57.1      |          |         |
| 10    | A/J          | α             | 1.0           | 15.3          | 75.0      | 41.4*    |         |
| 11    |              |               | 1.8           | 16.4          | 71.7      | 20.4*    | 14.3    |

Cells from normal mouse spleens were cultured and stained using FITC-anti-K^d or TRITC-anti-K^d. Total cell counts were determined as cells positive for membrane H-2K and the percentage of cells with intracellular H-2K was given by: [cells positive for intracellular H-2K/cells positive for membrane H-2K] × 100.

* Partners in three separate two-way MLR. Percentages refer to cells of one haplotype only.

Haplotype designations are those used in reference 34.
Figure 1. Immunofluorescence of intracellular Class I MHC molecules in Con A-activated lymphocytes. BALB/c cells were cultured for 3 d in Con A as described. (a and b) Cells were exposed in suspension to FITC-anti-K4 antibody at 0°C for 30 min, cytocentrifuged, fixed, and stained with TRITC-anti-K4. (c and d) Cells were exposed to FITC-anti-K4 at 37°C for 30 min, cytocentrifuged, fixed and stained with TRITC-anti-K4. (e and f) C3H/HeJ splenocytes cultured in Con A for 3 d were fixed in ethanol and stained with unconjugated goat anti-Con A and a TRITC-rabbit anti-goat Ig sandwich followed by FITC-anti-K4. Left and right frames are the same cells viewed with illumination specific for rhodamine (a, c, and e) and fluorescein (b, d, and f) respectively.
ified the intracellular H-2K-positive cells to be >95% Thy-1.2 positive and >97% slg negative. They were, therefore, T cells. Furthermore, intracellular H-2K containing vesicles were absent from cells stimulated with the B cell mitogen, LPS. As shown in Table I, lymphocytes positive for intracellular H-2K were observed in freshly isolated normal spleen cell populations. However, the percentage of such cells increased considerably in cultures stimulated with the T cell mitogen Con A or significantly as a consequence of allogeneic stimulation in a mixed lymphocyte culture (Table I).

Kinetic experiments as represented in Fig. 2, a and b show that expression of intracellular H-2K molecules is an event unrelated to the process of cell proliferation as such since, as already stated, LPS-treated lymphocytes proliferated quite as much as those stimulated by Con A (Fig. 2b), stained positively for membrane H-2K antigens but failed to express intracellular H-2K in significant quantities relative to control cultures. Furthermore, spleen lymphocytes stimulated with Con A showed a 10-fold increase in cells positive for intracellular H-2K by 24 h when the proliferative response was less than 1.5-fold. Therefore, this increase in cells with internal Class I MHC antigens cannot be explained as the selective expansion of previously existing subpopulations expressing intracellular H-2K. Rather, it can be taken to represent a cellular event related to the activation of spleen lymphocytes of T cell lineage.

The Intracytoplasmic Vesicles with Class I MHC Molecules are Formed by Internalization from the Plasma Membrane.

There are two obvious alternatives for the formation of intracytoplasmic vesicles with Class I MHC molecules.
sized MHC molecules previous to their insertion in the plasma membrane. Alternatively, the vesicles might be produced by internalization, from the plasma membrane, through a process of endocytosis. To resolve this alternative, experiments were performed in which cultures of mouse spleen cells, after 3 d of stimulation with Con A, were exposed to the continuous presence of FITC-conjugated monoclonal anti-H-2K antibodies and kept at 37°C. At different times after the beginning of the exposure to the antibodies, the cells were collected, cytocentrifuged, fixed, and restained with the same antibody to which they had been exposed in culture, but conjugated to TRITC instead of FITC. The rationale of this procedure was that, if the H-2K-containing vesicles were formed by internalization from the membrane, all those that had arisen after the exposure of the culture to the FITC-antibody should be labeled by fluorescein, whereas the pre-existing ones should be labeled with rhodamine, since we expected that the MHC antigenic sites that bound the FITC-conjugated monoclonal antibody in culture would not be available for binding of the same antibody (TRITC-conjugated) in the cytoplasm of the fixed cells. The fact that the particular antibody used completely competed with itself in such a system was supported by Fig. 1, a and b, which shows that membrane H-2K antigens stained by FITC-conjugated antibody when the cells were in suspension were not restained by the same antibody (TRITC-conjugated) after the cells were cytocentrifuged and fixed; also complete inhibition of the binding of the second conjugate (rhodamine) was observed if the fixed cells were first stained with the FITC-conjugate.

The results of such an experiment are shown as a function of time in Fig. 3, for cells sampled after 15 min of exposure of FITC-anti-Kª, practically all the cells that had intracytoplasmic H-2K (74% of all cells in this particular 3-d culture with Con A) showed variable numbers of fluorescein-containing vesicles. The same cells also showed other vesicles that bound TRITC-conjugated antibody when exposed to this reagent after fixation; a similar pattern, with an increase of the ratio of fluorescein-labeled to rhodamine-labeled vesicles was seen in cells sampled at 30 min (see Fig. 1, c and d). The fluorescein-positive vesicles were totally absent from control cells exposed to FITC-anti-H-2K for 1 h at 0°C (Fig. 1 a), at which temperature internalization could not take place (11). With progressive time of exposure to the FITC-antibody, under physiological conditions, the percentage of cells that showed “new” (fluorescein-labeled) vesicles remained equivalent (78 ± 3%) to the total number of cells which initially presented with internal H-2K, whereas the percentage of cells that had “pre-existing” H-2K vesicles (rhodamine-positive) progressively declined. This experiment has been repeated reversing the fluorochrome-anti-Kª antibodies used with identical results and shows that practically the entire population of the existing intracytoplasmic vesicles that contain Class I MHC molecules in activated T cells can be substituted, within a few hours, by comparable vesicles that continuously form by internalization from the plasma membrane. The conclusion is inescapable that the “pre-existing” H-2K-positive vesicles that were in the cytoplasm of the Con A stimulated cells before the exposure to the labeling antibody, were also formed by a process of internalization proceeding at a comparable pace.
In some cells we observed a few vesicles apparently marked by both labels; this might have been the consequence of an intracytoplasmic fusion of two vesicles or might simply have been due to the insufficient resolving power of the light microscope.

Internalization Does Not Depend upon Cross-linking of the MHC Molecules by Antibody. It appears that the process of internalization of Class I MHC molecules in stimulated T cells is "spontaneous" and does not depend upon cross-linking by a ligand such as antibody or Con A. Clearly the anti-MHC antibody was not involved since intracytoplasmic H-2K-positive vesicles were originally observed in cells never exposed to antibody (Table I). Even in cells exposed to anti-H-2K antibody, as shown in the preceding section, the antibody merely acted as a label of the H-2K molecules without affecting much vesicle turnover (further work on this problem is in progress in our laboratory). Even more important is the observation that exposure to anti-H-2K antibodies cannot induce the internalization of these molecules in those cells that do not spontaneously internalize them. This fact is shown in Table II where it is reported that overnight exposure...
of murine lymphoid cells to unconjugated anti–H-2K antibodies at 37°C resulted in a significant percentage of vesicles of internalized label only by cells that spontaneously express Class I MHC-positive vesicles.

**Internalization Does Not Depend upon Cross-linking of the MHC Molecules by Con A.** The lectin Con A reacts with the carbohydrate moiety of histocompatibility antigens (12). It is, therefore, conceivable that the internalization of Class I MHC molecules, in cells cultivated in vitro with Con A for prolonged periods of time, may be the consequence of a direct interaction of Con A with these molecules.

However, we stress the fact that we have observed Class I MHC internalization in cells not exposed to Con A (Table I), such as these mouse lymphoid cells subjected to allogeneic stimulation in MLR (13, 14). Note that Table I also shows an increase of intracellular H-2K-positive lymphocytes for untreated cells cultured over 3 d. This phenomenon might have been the consequence of syngeneic stimulation under in vitro culture conditions as reported for murine spleen lymphocytes (15, 16).

When we consider the cells stimulated by Con A, one fact against interpreting the H-2K internalization as due to a direct interaction of H-2K antigens with Con A is the observation that Con A–activated cells harvested, washed, and kept in the presence of 50 mM of the Con A inhibitor α-methylmannoside internalized H-2K molecules in a manner indistinguishable from that of control cultures treated with the same concentration of galactose (data not shown).

Furthermore, the increase with time of the percentage of cells with intracellular H-2K, as seen in Con A–treated cultures, is progressive over 3 d (Fig. 2a), whereas the maximal uptake of Con A is reported to occur by 24 h (17).

Actually, Con A itself, as expected, could be localized by immunofluorescence in the cytoplasm of the cells that had been exposed for 3 d to this mitogen. However, as shown in Fig. 1, e and f, although intracytoplasmic Con A and Class I MHC molecules could be found together in some vesicles, the majority of vesicles containing H-2K were clearly negative for Con A.

We would also like to mention an observation from our laboratory (Tse and Pernis, unpublished), namely that mouse L cell fibroblasts exposed to Con A

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**Table II**

| Mitogen | Percent cells with intracellular antibodies |
|---------|-------------------------------------------|
|         | Experiment 1* | Experiment 2† |
| None    | 3.8          | 3.1          |
| Con A   | 23.1         | 34.3         |
| LPS     | 0            | 0            |

BALB/c cells were cultured, exposed to unconjugated anti-Kd at 37°C for 18 h, and stained for membrane and intracellular mouse IgG. Percent cells = [cells positive for intracellular anti-H-2K/cells positive for membrane anti-H-2K] × 100.

* Part of Expt. 1, Table I.
† Part of Expt. 4, Table I.
accumulate the lectin in numerous intracytoplasmic droplets, but do not show any evidence of co-internalization of MHC antigens.

On the whole, we think that there is overwhelming evidence to support the statement that the internalization of Class I MHC molecules in mouse lymphoid cells stimulated by Con A is not the direct consequence of cross-linking of the MHC molecules by the lectin.

Selectivity of Class I MHC Internalization in Activated Lymphoid Cells. It is apparent that the process of Class I MHC internalization involves selection of those molecules to be internalized and is not simply the consequence of the formation of vesicles with a composition which reflects that of the plasma membrane.

Associated intracytoplasmic staining using directly conjugated anti-Thy-1.2 or anti-T200 with a TRITC-goat anti-rat Ig sandwich showed complete exclusion of both the T cell–specific Thy-1.2 membrane antigen and the nonpolymorphic surface lymphoglycoprotein T200 from H-2K–containing vesicles (data not shown). The anti-K\(^d\) monoclonal antibody 20-8-4S used in the current studies has been shown recently to detect a Qa-like cell surface antigen distinct from D and L gene products, which mapped to the right of H-2D (D. Sachs, personal communications). However, associated intracytoplasmic staining of Con A–activated A/J (K\(^k\), D\(^a\)) splenocytes using FITC-anti-K\(^k\) and TRITC-anti-K\(^a\) did not detect the presence of this antigen in vesicles containing H-2K.

Furthermore, Con A–activated lymphoid cells did not show any internalization of Class II MHC molecules, although membrane I-A–positive cells represented a considerable proportion of total cells in murine spleen cultures after 24 h in Con A; actually, they have been reported to express these molecules on the plasma membrane in increased amounts (18). Conversely, LPS-activated murine lymphoblasts internalize Class II MHC molecules (Buck and Pernis, submitted for publication) but, as we have indicated before, they do not show intracytoplasmic vesicles with Class I MHC antigens.

The Internalization Process is Blocked by Monensin and is Affected by Cycloheximide. The effect of cycloheximide on the expression of H-2K in Con A activated lymphocytes was investigated. As shown in Fig. 4a, inhibition of protein synthesis by this antibiotic produced a linear decrease in the expression of intracellular H-2K antigen resulting in total abolition of the intracellular compartment by 5 h.

In contrast, the expression of H-2K antigen on the cell surface shows a decrease over the first 2 h to 70% of control with no further changes upon continued exposure to cycloheximide. There was no significant decrease in cell viability over 5 h, but an increase in cell size was detected by light scatter as measured on the cell sorter.

Con A–activated lymphocytes treated with cycloheximide for 3 h presented a 70% reduction in the expression of intracellular H-2K on a cell to cell basis. However, exposing cells pretreated for 3 h with cycloheximide to FITC-anti-K\(^d\) at 37°C, as described in a previous section, showed that the reduced intracellular H-2K compartment in such cells can, nonetheless, be replaced over 1 h by antibody-labeled vesicles originating from the cell membrane.

Such observations are in contrast to studies using monensin (Fig. 4b). This
Figure 4. Effect of cycloheximide or monensin on the expression of H-2K and the internalization of anti-H-2K antibodies. BALB/c splenocytes cultured in Con A for 3 d were exposed to (a) 50 μM cycloheximide or (b) 25 μM monensin. After various times cells were analyzed for surface (closed circles) and intracellular (open circles) H-2K as described in Materials and Methods. Percent surface H-2K is given by: \[ \frac{\text{mean fluorescence per cell with pretreatment}}{\text{mean fluorescence per cell without pretreatment}} \times 100 \], and percent intracellular H-2K is given by: \[ \frac{\text{cells positive for intracellular H-2K with pretreatment}}{\text{cells positive for intracellular H-2K without pretreatment}} \times 100 \]. Cells with (+) and without (−) drug pretreatment for 3 h were analyzed for their capacity to internalize FITC-anti-Kd as described for Fig. 3. Percent fluorescein-positive cells (open bars) is given by: \[ \frac{\text{cells containing fluorescein positive vesicles with pretreatment}}{\text{cells containing fluorescein positive vesicles without pretreatment}} \times 100 \], and percent rhodamine positive cells (hatched bars) is given by: \[ \frac{\text{cells containing rhodamine positive vesicles with pretreatment}}{\text{cells containing fluorescein positive vesicles without pretreatment}} \times 100 \].
ionophore (19) has little significant effect on the expression of either membrane or intracellular H-2K. However, cells pretreated for 3 h with monensin before exposure to FITC-anti-K\textsuperscript{d} at 37°C showed an intracellular compartment that can no longer be replaced by newly formed vesicles. It appears that both the formation of new H-2K-containing vesicles and the disposal of the pre-existing ones are blocked by exposure of the cells to monensin. This drug has been reported to affect cells in several ways, including the inhibition of receptor recycling in other systems (20).

**Discussion**

It appears from our work that murine T lymphoid cells spontaneously internalize via an endocytic vesicular pathway, their Class I MHC molecules from the plasma membrane. The proportion of cells showing this process is considerably increased by stimulation of the spleen lymphocytes with a mitogen (Con A) or with allogeneic cells in mixed-lymphocyte reaction.

It is important to emphasize that the internalization process is spontaneous and does not require cross-linking by antibodies. Actually, antibodies directed against Class I MHC antigens do not appear to be able to induce internalization or modulation of these molecules in lymphoid cells that do not spontaneously internalize them. This is apparent from our own experiments as well as from previous work by Hutteroth et al. (21) where it was shown that antibodies directed against β\textsubscript{2} microglobulin were unable to determine the modulation of their target in human B lymphoblastoid cells; similar observations with murine B cells were made by Grey et al. (22). There is, on the other hand, some evidence that cross-linking by antibodies may determine internalization of Class I MHC antigens in nonlymphoid cells (23).

On the whole, the process of MHC internalization in activated lymphoid cells resembles more closely the spontaneous internalization of other receptors as seen in different kinds of cells, such as the low density lipoprotein (LDL) receptor in fibroblasts (24), the asialoglycoprotein receptor in hepatic cells, (25) or the receptor for transferrin in fibroblasts (26) and in lymphoblasts, (27) than the internalization of membrane immunoglobulins in B lymphoid cells, which never occurs spontaneously and is instead strictly dependent upon interaction with a cross-linking ligand (28).

The spontaneous internalization in different cells of the above-mentioned receptors is, in every case, part of a process of active recycling whereby the internalized molecules are rather rapidly returned to the plasma membrane. Considerable interest has recently been generated by this recycling of membrane components as a process of obvious physiological significance (29). It is indeed very likely that the internalization of MHC molecules in activated lymphoid cells is also part of a recycling process. If this were not the case, in consideration of the speed of the internalization process as shown by our kinetic studies (Fig. 3), the half-life of membrane Class I MHC molecules should be much shorter than it has been reported (30); in this respect it is noteworthy that activation of T cells determines an increase, rather than a decrease of the half-life of these molecules (31). Our experiments on the effects of monensin are in general
agreement with the concept of an active recycling of Class I MHC molecules in activated T lymphoid cells, a possibility that is also supported by experiments on the pH of the internalized vesicles recently performed in our laboratory (R. Murphy et al., submitted for publication).

In the pattern that is emerging, the specificity aspects are intriguing. Clearly, activated T cells do not internalize a representative sample of their plasma membrane, but select the molecules that are included in the vesicles: Class I MHC molecules are included, whereas Thy-1 and the lymphoglycoprotein T200 are excluded; we do not know as yet what else can be included with the Class I. Other cells, such as activated B lymphoblasts, develop different programs and internalize Class II MHC molecules in vesicles from which Class I MHC antigens are excluded.

This system appears different from that described for fluid phase pinocytosis in cultured macrophages (11); the mechanisms of this selectivity are unknown. Pearse and Bretscher (33) have hypothesized that selective recycling of different membrane molecules may be related to the expression on the membrane of specific adaptors that establish specific connections between the different membrane proteins and clathrin-coated pits where the incoming arm of the recycling process is started.

Another aspect of the selectivity of MHC internalization pertains to its relationships with cell differentiation. We have already noted the differences between lymphocytes and fibroblasts and between B and T immunocytes; another difference shown by our work is that between resting and activated T lymphoid cells. We have shown that this difference is not directly the consequence of cell proliferation; apparently it is related to the differentiation state of the cells and might involve the differential expression on the cell membrane of other molecules that associate with histocompatibility antigens. This problem is presently under study in our laboratory.

Considering our basic hypothesis that Class I MHC molecules are actively recycling in stimulated T lymphoid cells, we were surprised by the observation that inhibition of protein synthesis by cycloheximide progressively inhibited the internalization of these molecules. Actually, other recycling receptors such as the LDL receptor in fibroblasts are not affected by inhibition of protein synthesis and their internalization is not influenced by treatment with cycloheximide over a period of 48 h (32).

In view of our observations on the effects of cycloheximide, we reconsidered the possibility that the intracellular vesicles in activated T cells might actually contain newly synthesized Class I MHC molecules on their way to the plasma membrane, rather than MHC antigens internalized from the membrane. However, the "chasing" experiments showing that all the MHC-containing vesicles could eventually be labeled with the corresponding antibody did not leave any reasonable interpretation except that of the membrane origin of the intracellular molecules as visualized by immunofluorescence. It is noteworthy that a comparison of Figs. 3 and 4a shows that the decline of the percentage of cells still showing unlabeled ("pre-existing") vesicles after the exposure of the membrane MHC molecules to antibody is about twice as fast as the decline of the percentage of cells with intracellular MHC molecules in the samples treated with cyclohexi-
mide. Actually after 3 h of exposure to cycloheximide, a reduced percentage of cells was able to internalize labeled molecules from the membrane. This provides direct demonstration that the progressive fall of cells showing intracellular H-2K after a block of protein synthesis was due to a decline in the internalization process and argues against the fact that the intracellular vesicles are transport organelles for MHC molecules synthesized in the endoplasmic reticulum that have not as yet been displayed on the cell surface. We hypothesize that, unlike the internalization of other receptors in other cells, the spontaneous endocytosis of Class I MHC molecules in activated T lymphoid cells requires the availability of a short-lived polypeptide that is depleted in a few hours after exposure of the cells to cycloheximide. An alternate possibility might be that only newly synthesized MHC molecules can be internalized shortly after their insertion on the plasma membrane. We consider this as rather unlikely.

In conclusion, we have demonstrated that Class I MHC antigens in activated T lymphoid cells are engaged in an active process of spontaneous internalization which generates numerous intracytoplasmic vesicles containing these molecules. It is likely that this process is part of a recycling pattern not dissimilar to that recently described for other membrane receptors in other cells. The physiological significance of such a process is not, however, as clear as the metabolic relevance of, for instance, the internalization of the receptors for LDL or the Fe-carrier transferrin. The established function of MHC molecules in immune reactions is one of recognition by T cells where they serve as elements of stimulation in an allogeneic system or restriction in a syngeneic system. It is not obvious why activated T lymphocytes should mobilize their own Class I MHC molecules or how this process might be related to the immune function of these cells, except for the general possibility that it may be involved in the recognition of one T cell by another in events where specificity is provided by antigens or the cell's own idiotypes. It is reasonable to expect that an understanding of the functional significance of the process of Class I MHC recycling in T cells will be greatly facilitated by the study of the biochemical events involving these molecules which take place in the intracytoplasmic vesicles.

Summary

A low proportion of T lymphocytes in normal mouse spleen contains small intracytoplasmic vesicles showing Class I MHC molecules. After stimulation in vitro in a mixed lymphocyte reaction or by addition of Con A, the proportion of T cells with such intracytoplasmic vesicles increases progressively and becomes the majority.

Labeling with fluorochrome-conjugated antibodies has shown that the vesicles are formed by internalization of molecules from the plasma membrane. The process is spontaneous and does not require cross-linking by antibodies or other ligands; it is selective inasmuch as other molecules (Thy-1 and T200 antigens) are not included and it is specific since it is not performed by other cells such as B lymphoid cells or fibroblasts.

On the whole the process shows similarities with the internalization and recycling of other receptors, such as the receptors for different macromolecules of metabolic or informational significance, as seen in other cells. On the other
hand, the specificity of Class I MHC mobilization in T lymphoid cells suggest a role for this process which is related to the immune function of these molecules.

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