A Subset of CD4+ Memory T Cells Contains Preformed CD40 Ligand That Is Rapidly but Transiently Expressed on Their Surface after Activation through the T Cell Receptor Complex

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Summary

Signaling through surface CD40 is essential for selecting B cells that have mutated their immunoglobulin variable region genes in germinal centers and is an important signal in the early stages of antibody responses to T cell-dependent antigens. It is shown that a subset of CD45RO+, CD4+ T cells isolated from human tonsil contains preformed 30–35-kD ligand for CD40. This is expressed on their surfaces within 5 min of their antigen–receptor complexes interacting with CD3ε antibodies bound to ox erythrocytes. This surface expression does not require de novo protein synthesis and lasts for only 1–2 h. Preformed CD40 ligand (CD40L) was not detected in any CD4+ CD45RA+ T cells, but >90% of all CD4+ T cells from the tonsil can be induced to express large amounts of CD40L on culture with phorbol myristate acetate and the calcium ionophore ionomycin. This expression of CD40L starts between 1 and 2 h, peaks at 6 h, and remains at a high level for >20 h. It is totally prevented by adding a concentration of cycloheximide that inhibits CD25 synthesis by these activated cells. While CD3ε antibody bound to ox red cells is a good inducer of surface expression of CD40L, it is a much less potent inducer of CD40L synthesis than phorbol myristate acetate with ionomycin. Immunohistological analysis of tonsil sections shows that cells containing CD40L are located mainly in the outer zone of germinal centers and the margins of the T zones that are rich in dendritic cells (interdigitating cells). The distribution of these cells is consistent with: (*a*) their interaction in T zones with B cells that have taken up and processed antigen and (*b*) their involvement in B cell selection in germinal centers.

Germinal centers are a site of rapid and extensive expansion of B cell clones activated during responses to protein-based Ag (1, 2). The members of these clones undergo somatic mutation in their Ig V region genes (3) and appear to be selected subsequently on the basis of their specificity for Ag held on local follicular dendritic cells (4). Selected cells leave the germinal center either as memory B cells (5), or plasmablasts (6). Tonsil germinal center B cells, isolated and cultured in standard tissue culture medium, undergo death by apoptosis within a few hours, but this can be delayed if their surface Ig is cross-linked and further protection from apoptosis is achieved on exposure to the 30–35-kD CD40 ligand (CD40L)[1] (4, 7). CD40L can be expressed on the surface of T cells during cognate interaction with B cells or dendritic cells (8–10). This opens the possibility that the germinal center B cells, centrocytes, pick up native Ag held on follicular dendritic cells, process it, and present it to local T cells that are then induced to express CD40L (11). It has been reported that centrocytes can take up Ag from follicular dendritic cells, process it, and present it to T cells (12). Experiments in which CD40L interaction with CD40 is blocked in established germinal centers in mice indicate that CD40 is required for memory B cell formation (13).

Both B cells and dendritic cells in the T zones of secondary lymphoid organs constitutively express CD40 (14). This opens the possibility that T cell priming and extrafollicular T cell–B cell interactions involve signaling through CD40 and CD40L. Inhibition of this signaling pathway in mice using Ab against CD40L markedly impairs primary T cell-dependent Ab responses (15), as well as germinal center formation (16). An immunohistological analysis of human secondary lymphoid tissues, using a mAb against human CD40L, identified CD40L in germinal centers (17), but studies in mice have reported CD40L expression in T zones, but not in germinal centers

[1] *Abbreviation used in this paper:* CD40L, CD40 ligand.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/04/1293/09 $2.00
Volume 181 April 1995 1293-1301

(15). The present study has investigated CD40L expression and the induction of its expression by human tonsil T cells. It indicates that CD40L is present inside a minority of $CD45RO^+$, $CD4^+$ T cells, in the T zones and is present in many germinal center T cells. T cells with preformed CD40L can be induced to express this rapidly on their surfaces by signaling through their TCR complexes.

Materials and Methods

Tonsils and the Preparation of Tonsil Cell Suspensions. Human tonsils were obtained from patients undergoing tonsillectomy to relieve obstruction to the respiratory passages and improve drainage of the middle ear. The age of these patients ranged from 5 to 27 yr. All of the tonsils studied were histologically normal. Pairs of tonsils were carefully teased, the mononuclear cell suspension was layered onto Ficoll-Isopaque (Pharmacia, Milton Keynes, United Kingdom) and centrifuged at 450 *g* for 20 min at 20°C, and the interface cells were washed in RPMI 1640 (GIBCO, Paisley, United Kingdom).

Antibodies and Fluorochromes. The following mAbs, after coating on ox RBC, were used for negative selection procedures or to activate T cells: BU12, CD19 (14); GC6, CD45RA (The Binding Site, Birmingham, United Kingdom); B941, CD8 (a gift from Claud Mawas, Marseille, France) (18); UCHT1, CD3 (a gift from Peter Beverley, University College Hospital, London) (18). These antibodies were attached to ox RBC, using the chromic chloride technique (19). The coatings involved suspending 2×10^9 ox RBC in 100 μl saline containing 0.3 mg of IgG preparation of mAb. To this, 60 μg of aged $CrCl_3$ was added in 600 μl saline. The mixture was then incubated overnight at 4°C before washing the cells and resuspending them in RPMI 1640. Fresh cell coatings were made for each experiment. When $>2 \times 10^9$ ox RBC were used, replicate batches of coated cells were prepared.

The following conjugated mAbs were used for FACScan® (Becton Dickinson, Oxford, United Kingdom) analysis, CD3-PE and CD57-FITC (Becton Dickinson), CD45RA-PE (Serotec Ltd., Oxford, United Kingdom), CD19-FITC, CD4-FITC, CD8-PE, CD25-FITC, CD45RO-PE (Dako, High Wycombe, United Kingdom). The five different mAbs against CD40L used were: 5c8 (a gift from Seth Lederman, Columbia University, New York) (10), 5B4 (anti-TRAP1) (a gift from Richard Kroczek, Robert Koch Institute, Berlin) (9), M90, M91, and M92 (gifts from Richard Armitage, Immunex, Seattle). Recombinant CD40L (20) was also a gift from Richard Armitage. Recombinant human CD40 linked to human Fcμ heavy chain was a kind gift from Dr. Peter Lane of the Basel Institute of Immunology (21). Both 5B4 and M90 were biotinylated, and GC6 was labeled with FITC, as described in (22). The following fluorescent detection reagents were used: streptavidin-PE (Becton Dickinson), sheep anti-mouse IgG-FITC (Sigma Chemical Co., Poole, United Kingdom). For immunohistology, in addition to the five anti-CD40L mAbs, mAbs against the following specificities were used: Ki67 (23), mAb MIB1 (a gift from Johannes Gerdes, Borstal, Germany): CD45RO, mAb UCHL1 (a gift from Peter Beverley) (24), and CD4, mAb MT310 (Dako). Rabbit anti-mouse Ig and alkaline phosphatase mouse anti-alkaline phosphatase complex (Dako) were used to detect the binding of these Abs.

Cell Fractionation and Phenotypic Analysis of Isolated Cells. The tonsil cell suspension was submitted to negative selection using a direct rosetting technique with mAb-coated ox RBC. To isolate $CD4^+$ T cells, ox RBC coated with CD19 and CD8 mAbs were used. Ox RBC coated with mAbs against CD45RA and CD8 were used to isolate $CD4^+$, $CD45RO^+$ T cells. The procedure was set up to obtain high cell purity, rather than high yields. Between 5×10^8 and 10^9 tonsil cells were incubated with 2×10^9 ox RBC coated with CD19 mAb for 20 min, then centrifuged onto Ficoll-Isopaque for 20 min before the nonrosetting cells were harvested from the interface. These cells were then either incubated with 4×10^8 ox RBC cells coated with CD19 mAb and 4×10^8 ox RBC cells coated with CD8 mAb to isolate $CD4^+$ T cells, or with the same numbers of CD45RA and CD8 mAb–coated ox RBC to isolate $CD4^+$ $CD45RA^{low/-ve}$ T cells. Again, the nonrosetting cells were separated by density centrifugation. The cells separated with CD19 and CD8 mAb–coated RBC to isolate $CD4^+$ T cells were subjected to two rounds of rosetting, and the final cell yields were between 1.3 and 1.8×10^7. More efficient separations were obtained with negative selection using CD8 and CD45RA mAb–coated RBC for which only one round of rosetting was required, and the final cell yields were between 1.9 and 3.0×10^7. After the rosetting procedure and gradient centrifugation, the interface cells were washed and resuspended in RPMI 1640 supplemented with 10% FBS (GIBCO). All the procedures were performed at 4°C, except centrifugation of cells using Ficoll-Isopaque, which was carried out at 20°C. The purity of the different isolated T cell subsets was assessed by FACScan® analysis as described below.

Culture Conditions. Freshly isolated T cells were incubated in 96 flat-bottom–well plates at a concentration of 5×10^5 cells/ml. The cells were cultured with two different conditions: with PMA at 1 nM (Sigma Chemical Co.) and ionomycin 0.7 μg/ml (Calbiochem-Novabiochem, Beeston, United Kingdom), or with CD3ϵ mAb-coated ox RBC. These latter ox RBC were added at a ratio of 10 red cells per lymphocyte (having been determined as the ratio that gave optimal expression of CD40L). Cycloheximide (Sigma Chemical Co.) was used to study de novo protein synthesis. The dose used (10 μg/ml) was identified as the minimum dose needed to produce >95% inhibition of CD25 expression by T cells activated by PMA with ionomycin. Cells were preincubated with cycloheximide for 2 h before adding PMA with ionomycin or CD3ϵ mAb–coated ox RBC, and it was maintained in the cell cultures during the total culture time.

Phenotypic Analysis. Cell suspensions were stained by direct or indirect immunofluorescence by washing them in PBS supplemented with 5% goat serum (GIBCO), after which 2×10^5 cells were incubated with the appropriate dilution of the first Ab (2 Abs for double immunofluorescence staining) for 30 min on ice. After two washes the cells labeled with direct conjugates were fixed with 1% formaldehyde (Sigma Chemical Co.). Cells labeled with biotinylated 5B4 or M90 mAb were incubated with streptavidin-PE, and cells labeled with 5c8 were incubated with sheep anti–mouse IgG FITC for a further 30 min before washing and fixing. Cells were analyzed within 48 h of staining using a FACScan® analyzer (Becton Dickinson).

Immunohistology. 4-μm acetone-fixed serial cryostat sections of tonsils were stained to reveal CD40L expression. The immuno-alkaline phosphatase technique was used, as described in reference 25. Briefly, the sections were incubated with the optimal dilutions of Ab for 1 h in a moist chamber, and were then thoroughly washed before a rabbit anti–mouse Ig was added for 45 min. After further washing, an alkaline phosphatase–conjugated mouse anti–alkaline phosphatase complex was added for 30 min. To increase the sensitivity of the technique, these two last steps were repeated twice, and, finally, the bound alkaline phosphatase was developed by

naphthol AS-MX phosphate (Sigma Chemical Co.) and Fast Blue BB salt (Sigma Chemical Co.). Endogenous alkaline phosphatase activity was blocked with levamisole.

Quantitation of the number of T cells with a particular phenotype per unit area was carried out using the point counting technique of Weible (26). Slides carrying 4-μm sections were viewed at a magnification of 250 with a 1-cm square graticule divided into 11 vertical and 11 horizontal lines, each 10-mm from the other, located in one eyepiece. The area of the section was estimated by the number of line intercepts that fell on the area being assessed, and the number of cells in that area were counted. An intercept will fall, on average, onto each 0.4 mm^2 of section. The frequency of cells in a particular compartment is expressed as the average number of cells per square millimeter of that compartment. The following compartments were identified: (*a*) the T zone that was taken to be that area outside follicles that contained confluent or near confluent $CD4^+$ T cells; (*b*) the light zone of germinal centers that was identified as the area within germinal centers that did not contain confluent $Ki67^+$ B cells (27); (*c*) the outer zone that is defined in this study as a band of $CD4^+$ T cells located as a layer at the outer edge of the light zone.

Results

Induction of CD40L Expression on the Surfaces of $CD4^+$ T Cells. Previous studies have shown that the phenotype of almost all T cells found in tonsil germinal centers are $CD4^+$ $CD45RO^+$ and fail to express Bcl-2, a minority express CD57 (28, 29). Initial experiments studied all $CD4^+$ T cells that were prepared by negative selection of tonsil cell suspensions, $CD8^+$ and $CD19^+$ cells being removed. The phenotype of these negatively selected cells in a representative experiment is shown in Fig. 1. The mean level of $CD8^+$ cell contamination ± SD in 10 experiments was 0.51 ± 0.6%, and $CD19^+$ cell contamination was 5.0 ± 5.3. Most $CD4^+$ tonsil T cells were found to express CD45RA without CD45RO, or CD45RO without CD45RA. Unlike human peripheral blood T cells, relatively few tonsil $CD4^+$ T cells were found to express both these CD45 isoforms. The proportion of $CD4^+$ $CD57^+$ tonsil cells expressing CD45RO was assessed in four experiments. Again, $CD8^+$ and $CD19^+$ cells were removed by negative selection. In the four experiments, 99.2, 98.3, 99.8, and 99.9% of the $CD57^+$ cells were $CD45RO^{high}$. Very few of these cells coexpress CD45RA (Fig. 1).

None of the $CD8^-$ $CD19^-$ tonsil T cells isolated were found to express CD40L on their surfaces constitutively, but, on culture in 1 nM PMA plus 0.7 μg/ml ionomycin, a small proportion of cells started to express low levels of this protein on their surfaces within 15 min (Fig. 2). All of the cells expressing CD40L at this time were $CD45RA^{low/-ve}$. This level of expression remained constant for 1 h, but between 1 and 2 h most $CD4^+$ cells, including $CD45RA^+$ cells, had started to express CD40L. After 2 h, there was a rapid increase in the level of surface CD40L expression, peaking at 6 h when levels were 10–20 times those seen at 15 min (Fig. 2). Double staining for CD57 (Fig. 1) shows that a median of 5% (range 4–11%) of the $CD4^+$ tonsil T cell fraction expressed CD57. Most of these cells were induced to express surface CD40L within 15 min of activation with PMA plus ionomycin. All of the $CD57^+$ cells expressed CD40L after 20 h culture with PMA plus ionomycin. During these time course experiments, there was no significant alteration in the relative proportions of $CD45RA^{high}$ cells and $CD45RA^{low/-ve}$ cells (Fig. 2). In five experiments, in which the starting proportion of $CD45RA^{low/-ve}$ cells was 63, 67, 50, 37, and 45%, after 20 h culture with PMA and ionomycin, the respective alteration in the percentage of $CD45RA^{low/-ve}$ cells was +3, −10, +18, +2, and −2%.

Evidence that CD40L Is Contained Within a Subset of $CD4^+$, $CD45RA^{low/-ve}$ T Cells. To assess the requirement for de novo synthesis of CD40L for its surface expression, the experiments described in the previous paragraph were repeated in the presence or absence of 10 μg/ml cycloheximide. This was shown to be the minimum concentration of this agent that caused >95% inhibition of new CD25 expression by tonsil T cells cultured with PMA plus ionomycin. The cycloheximide was added 2 h before the addition of PMA and ionomycin and was present throughout the culture. Fig. 3 shows that the expression of CD40L induced by PMA and ionomycin on $CD45RA^{low/-ve}$ tonsil T cells at 15 min is not inhibited by the presence of cycloheximide. The second phase of CD40L expression by $CD45RO^+$ cells, starting 1 h after the addition of PMA plus ionomycin (Fig. 3 *A*), is totally inhibited in the presence of cycloheximide (Fig. 3 *B*). The

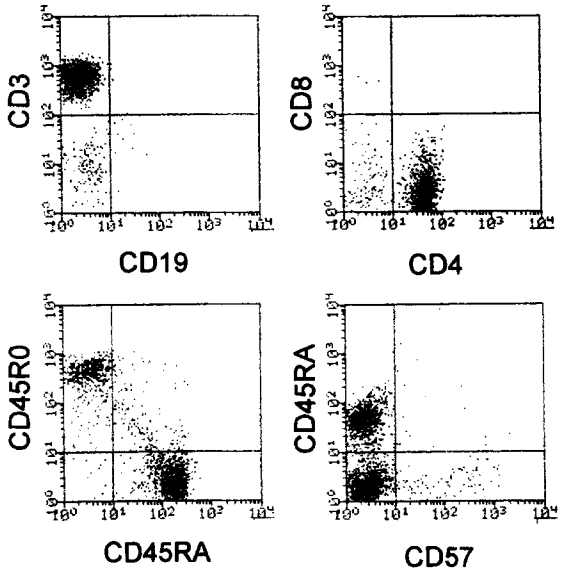

Figure 1. The phenotype of $CD8^-$, $CD19^-$ T cells isolated from human tonsil. The results are shown as FACScan® dot plots of the isolated T cells. The percentage of cells in the appropriate quadrants of the dot plots shown are: $CD3^+$, $CD19^-$ 90%; $CD4^+$, $CD8^-$ 92%; $CD45RO^+$, $CD45RA^-$ 48%; $CD45RA^+$, $CD45RO^-$ 44%; and $CD57^+$, $CD45RA^-$ 7%. Fluorescence intensity is plotted on a log scale. The cells shown on these dot plots are those contained within the gate containing viable small and large lymphocytes, as assessed by forward and 90° light scatter.

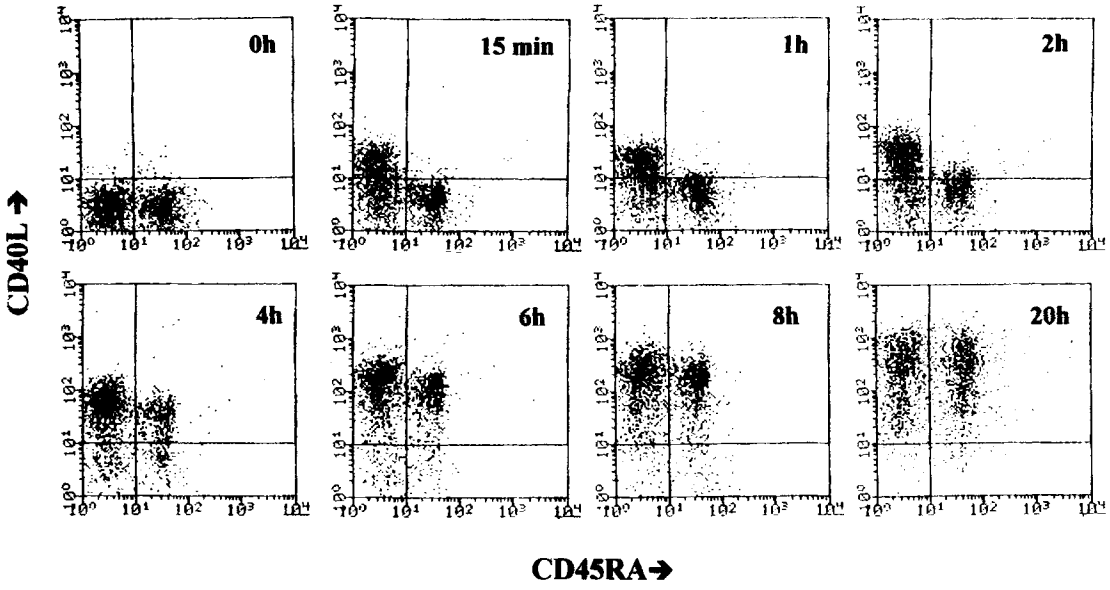

Figure 2. Induction of surface CD40L expression on T cells at intervals after activation with PMA and ionomycin. Cells were stained with a CD45RA-FITC mAb and a biotinylated CD40L mAb, which was revealed with streptavidin-PE. A subset of CD45RA$^-$ T cells express low levels of CD40L by 15 min. This level of CD40L expression by a subset of CD45RA$^-$ T cells remains relatively constant during the first hour of culture. By 2 h, most cells in both the CD45RA$^-$ and the CD45RA$^+$ T cell subsets express some surface CD40L. The level of surface CD40L expression increases to reach its maximum by 6 h.

expression of CD40L by CD45RA^{high} cells, starting only 1 h after the addition of PMA and ionomycin (Fig. 3 *A*), is also totally inhibited in the presence of cycloheximide (Fig. 3 *B*). These experiments were analyzed using TRAP1, 5c8, and M90 mAbs against CD40L. Each Ab gave comparable results. The CD40L expression detected by these antibodies was totally blocked in the presence of excess recombinant CD40L.

Activation of CD45RA$^-$CD8$^-$ Tonsil T Cells through their T Cell Receptor Complexes Induces the Surface Expression of Preformed CD40L but Relatively Little New Synthesis of CD40L. In further experiments, CD8$^-$ CD45RA$^-$ cells were cultured with PMA plus ionomycin or ox RBC coated with mAb against CD3ε. Early expression of surface CD40L was also induced by interaction with CD3ε-coated ox RBC. This was not altered by the presence of 10 μg/ml cycloheximide (Table 1). When the cells were stimulated through CD3ε, the second cycloheximide-sensitive phase of CD40L expression was considerably less marked than it was when cells were activated using PMA with ionomycin. Far fewer cells were induced to express CD40L, and the duration of expression was much shorter. A further set of three experiments was carried out to assess the rate of early expression of surface CD40L. Both PMA with ionomycin and ox RBC coated with mAb against CD3ε induced surface CD40L within 5 min of adding these activators. The mean ± SD levels of surface CD40L expressed after 5 and 15 min were: 47.5 ± 9.4, and 51.3 ± 10.2 for activation using PMA with ionomycin and 16.8 ± 3.3, and 16.4 ± 8.5 for cultures where ox RBC coated with mAb against CD3ε were added.

Location of Cells Containing CD40L in Tonsil Sections. Serial frozen sections from each of three tonsils were studied for the expression of CD40L, CD45RO, and CD4 by tonsil T cells. The dark zone of the germinal centers was identified by staining for the proliferation-associated nuclear Ag Ki67. The average frequency of CD4$^+$ cells, CD45RO$^+$ cells, and CD40L$^+$ cells in the apical plus basal light zones and the outer light zone of germinal centers, and in the T zones in three tonsils is shown in Table 2. Fig. 4, *a–d* are photomicrographs of four serial tonsil sections, showing a secondary follicle and an area of T zone stained respectively, for CD40L, Ki67, CD45RO, and CD4 expression. The outlines of the zones are shown in Fig. 4 *e*. Fig. 4 *f* is a high magnification photomicrograph showing the granular nature of the CD40L staining. Cells with CD40L are seen both inside and outside germinal centers, but are most frequent in the outer zone of germinal centers. This zone also contains more CD45RO cells per unit area than the T zones. CD40L$^+$ cells were much less frequent in the rest of the light zone. Cells with CD40L appeared to be more frequent at the edge than in the heart of T zones (Fig. 4 *a*). As expected, the frequency in germinal centers of CD4$^+$ and CD45RO$^+$ cells counted in serial sections was the same, confirming that these molecules are coexpressed on germinal center T cells. Similar CD40L staining was obtained with the 3 mAbs: 5c8, M90, and M91. The mAb M90 gave the strongest staining. When excess soluble human CD40 linked to human Fcμ (21) was mixed with the 5c8, M90, or M91 added to the sections, staining was blocked. It has previously been reported by Lederman et al. (17) that the 5c8 does not bind to the CD40L that has already engaged CD40. Comparative blocking experiments were carried out indicating that soluble CD40 also

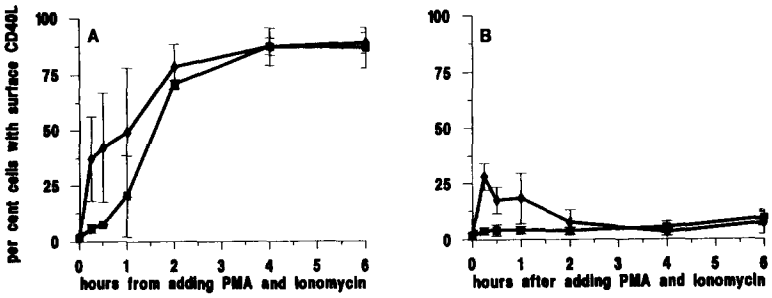

Figure 3. The surface CD40L expression by the CD45RA$^-$ and the CD45RA$^+$ T cell subsets induced by PMA and ionomycin, and the dependence of this effect of activation on protein synthesis. The mean ± SD expression of surface CD40L by CD45RA$^+$ cells (■) and CD45RO$^+$ cells (◆) in cultures: (*a*) without the inhibitor of protein synthesis, cycloheximide; (*b*) in which 10 μg/ml cycloheximide was added 2 h before the addition of PMA and ionomycin, and was present throughout the culture. These data are based on four experiments.

competes with M90 and M91 for binding to T cells expressing CD40L. Tonsil T cells treated for 18 h with PMA plus ionomycin were stained by indirect immunofluorescence with 5c8, M90, and M91 alone and in the presence of dilutions of soluble recombinant CD40. Staining with M91 was completely blocked by recombinant CD40 and staining with 5c8 or M90 was blocked by more than 10-fold. These findings support the conclusion that most CD40L is inside T cells in germinal centers, since the large amount of CD40 expressed by germinal center B cells would be likely to block the 5c8 binding sites on CD40L expressed on the surface of germinal center T cells (14). The level of CD40L expression detected by immunohistology was relatively weak and uniform. Isolated or clustered strongly positive cells were not seen, suggesting that high level expression induced by PMA and ionomycin in vitro may not reflect physiological expression in vivo.

Discussion

Signaling through CD40 and CD40L interaction has been reported to be necessary for the initiation of T cell–dependent Ab responses in mice (15). During the early phase of responses to protein-based Ag, there seems to be a requirement for T cells to interact sequentially with two cell types that have taken up and processed Ag. First, they must interact with dendritic cells and, then, with B cells; the first interaction priming the T cells (reviewed in reference 30), and the second providing Ag-specific activation signals to the B cell. It is unclear whether both of these specific interactions involve the transient expression of CD40L by the T cells at the site of cognate interaction. Both B cells and dendritic cells constitutively express CD40 (14), suggesting that this might be the case. On the other hand, further signals delivered during specific T cell activation seem to act at one stage and not the other. Thus, when T cell–B cell signaling through CD28/CTLA4 interaction with B7-1/B7-2 was blocked in vivo, T cell priming was unaffected, but the early stages of T cell–dependent B cell activation were markedly impaired (31). Dendritic cells are markedly more efficient than activated B cells in priming virgin T cells (32, 33), and evidence has been presented indicating that, under certain conditions, cognate interaction of virgin T cells with B cells in-

Table 1. *Induction of Surface CD40L Expression by CD45RA$^-$ CD8$^-$ T Cells with Anti-CD3–coated Ox RBC or PMA and Ionomycin, with and without Added Cycloheximide*

	Experiment 1				Experiment 2				Experiment 3			
	anti-CD3 ox RBC		PMA + ionomycin		anti-CD3 ox RBC		PMA + ionomycin		anti-CD3 ox RBC		PMA + ionomycin	
		cx		*cx*		*cx*		*cx*		*cx*		*cx*
0 min	2	2	1	1	2	2	0	0	2	2	1	1
15 min	7	4	43	24	26	48	49	59	14	13	23	34
1 h	18	17	43	7	38	57	nd	nd	20	23	25	28
2 h	nd	nd	nd	nd	66	31	70	3	31	13	65	5
4 h	29	8	89	7	47	14	98	5	17	7	82	2
6 h	nd	nd	nd	nd	7	10	94	9	6	5	91	8
20 h	10	1	95	7	1	3	48	2	0	0	63	0

The values shown are the percent cells expressing CD40L at the time intervals indicated after the addition of PMA and ionomycin or CD3ε mAb–coated ox RBC. The cultures represented in the columns headed cx had 10 μg/ml cycloheximide added 2 h before the activation signals. The level of contamination of the CD45RA$^-$ CD8$^-$ T cells with CD45RA$^+$ cells was 10.2, 0.25, and 0.3% in experiments 1, 2, and 3, respectively, and contamination with CD8$^+$ cells was <1% in each experiment.

Table 2. *Frequency of Cells Expressing CD40L, CD45RO, or CD4 in Different Tonsil Compartments*

T cell marker assessed	Outer light zone of germinal centers	Remainder of light zone	T zone
CD40L	4.4 ± 1.8	0.6 ± 0.2	2.0 ± 1.1
CD45RO	7.0 ± 3.4	2.0 ± 1.0	4.9 ± 0.4
CD4	7.3 ± 3.6	2.0 ± 1.1	13.5 ± 4.0

The results are expressed as the number of cells expressing the molecule indicated per square millimeter ± SD of 4-μm-thick tonsil section. Observations are based on the analysis of at least 250 cells in each area of three different tonsils. The light zone is defined as that part of the germinal centers where Ki67$^+$ cells are not confluent. In this study, the limit of the outer light zone is defined by the band of T cells found in this compartment that occupies the part of the germinal center immediately inside the follicular mantle (see Fig. 4). A full description of tonsil germinal center compartments is given in reference 27.

hibits the T cells' activation (33). It will be interesting to see if there is a difference in the ability of dendritic cells and B cells to induce T cells to store preformed CD40L.

T cell priming is associated with a switch from predominant CD45RA expression to the expression of CD45RO (24, 34–36). It is unclear from the present study if this is also accompanied by constitutive expression of intracellular CD40L, as only 40% of CD45RO$^+$ cells in the T zones were found to contain CD40L. Kinetic studies of the acquisition of intracellular CD40L expression during T-dependent responses in vivo are required. Induction of the expression of surface CD40L by both the CD4$^+$ CD45RA$^+$ and the CD4$^+$ CD45RO$^+$ T cell subsets has been described previously (37). The kinetics of induction of surface CD40L expression by PMA and ionomycin in these two T cell subsets was reported to be similar, but time points before three hours were not studied. In the experiments reported here, major differences between surface CD40L expression by CD45RO$^+$ and CD45RA$^+$ cells were only seen in the first two hours after activation. These differences were made considerably more obvious when protein synthesis was inhibited. Different activation requirements for CD45RO$^+$ and CD45RA$^+$ Th cells have been described by several authors (reviewed in reference 38). Ag-specific activation of CD4$^+$

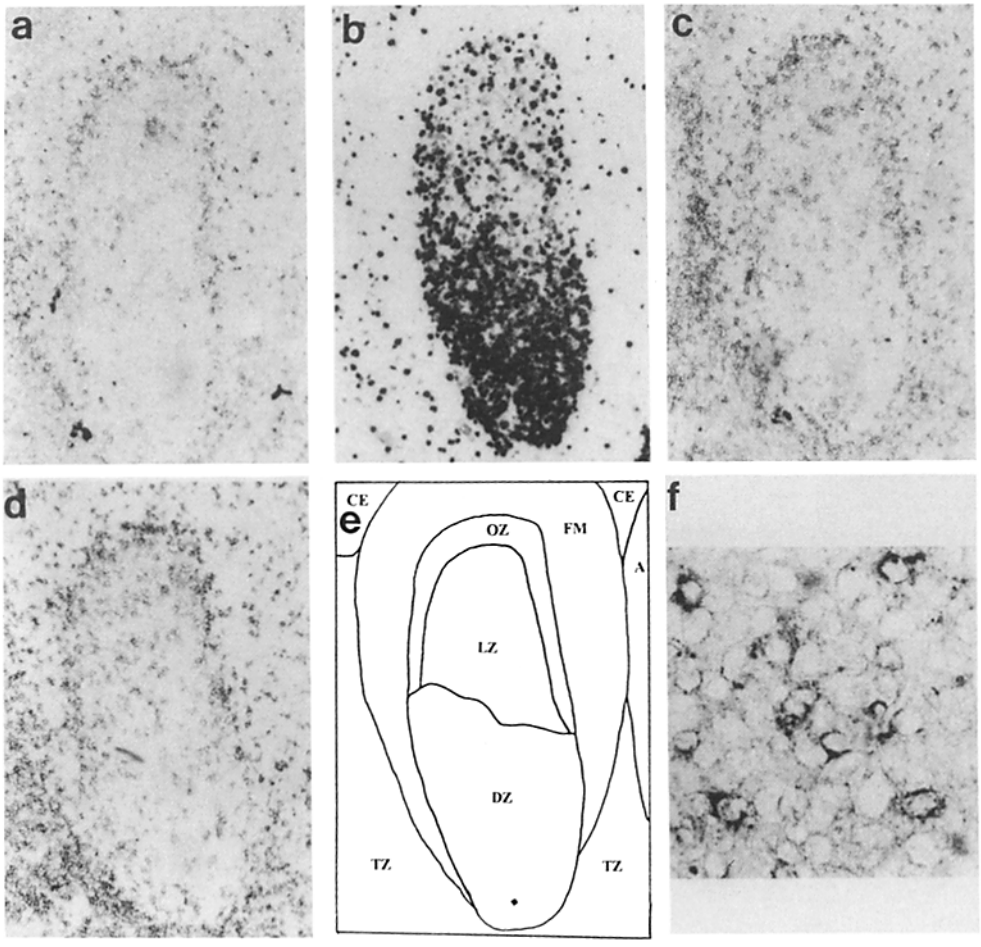

Figure 4. The location of cells containing CD40L in tonsil sections. CD40L staining, using mAb M90, is shown in the photomicrograph *a*. Figs. *b–d* are further photomicrographs of the same area of serial sections of the same tonsil stained, respectively, for expression of Ki67 (*b*), CD45RO (*c*), and CD4 (*d*). *e* identifies the areas in the serial tonsil sections: dark zone (DZ), light zone (LZ), and outer zone (OZ) of a germinal center. The FM is the follicular mantle surrounding the germinal centre, and A is part of the follicular mantle of an adjacent follicle. TZ is part of a T cell–rich zone, and CE is crypt epithelium. Note how the highest frequency of cells with CD40L in *a* is found in the outer zone and the outer part of the T zone that abuts onto the follicular mantle. *f* shows the granular nature of the CD40L staining. (*a–e*) ×100, (*f*) ×500.

$CD45RO^+$ T cells can be produced by B cells, but the $CD45RA^+$ $CD4^+$ T cells seem to need the action of professional APC (38, 39). The phenotype of $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells differ in the expression of the adhesion molecules CD11a, CD29, CD2, and CD44 (these being at lower levels on the $CD45RA^+$ T cell subset [40, 41]). Differences in the expression of these molecules may explain, in part, the different activation requirements for both $CD4^+$ T cell subsets (42). In addition, in memory T cell lines, the TCR complex appears to incorporate CD4 and CD45 isoforms; these act as three distinct entities in virgin cells, rendering them far less sensitive than memory cells to activation through their TCR (38, 43, 44).

B cell activation in the T zone results in the production of short-lived plasma cells (2) and B blasts that migrate to follicles to form germinal centers (45, 46). Interference with CD40L expression, using Ab against CD40L in vivo, inhibits the formation of germinal centers (16), although it is not clear if this inhibition is at the level of the T zone reaction, or germinal center formation, or both. Recirculating B cells (47) and newly produced virgin B cells (48) migrate into the outer parts of the T zone of the spleen and lymph nodes of rodents. The recirculating cells move on from there to the follicular mantles. The migration patterns of B cells into secondary lymphoid tissues in humans is not known. Assuming this is similar to that in rats, their migration pathway would pass through the outer T zones in the tonsil where CD40L-containing T cells are concentrated. Ag-specific migration of memory B cells from the marginal zone of the spleen to the outer T zones has also been described (2).

The speed at which T cells can express surface CD40L may be crucial in germinal centers; kinetic studies indicate that centrocytes either leave the light zone within 7 h or die by apoptosis in situ (2, 49). The present study shows that cells with preformed CD40L that can express this rapidly on their surfaces are present in the outer zone of germinal centers. The presence of $CD4^+$ $CD45RO^+$ T cells, both in the T zones of the tonsil and at the outer edge of germinal centers, has been noted previously (50).

In the present studies, it was found that when preformed CD40L was expressed on the cell surface, it was downregulated within 2 h (Fig. 3 *B*). Yellin et al. (51), in a recent report of a study of surface CD40L turn-over, indicate that interaction with CD40 induces rapid endocytosis of surface CD40L, thus limiting the length of surface expression of this molecule. Our failure to find constitutive surface expression of CD40L is consistent with the concept that this molecule is only expressed in secondary lymphoid tissues at the site of cognate interaction. It seems important that bystander cells should not gain access to CD40L expressed by their neighbors if cells that acquire autoreactivity through hypermutation of their Ig V region genes are to be eradicated.

The authors wish to thank S. Lederman, R. Armitage, R. Kroczek, P. Beverley, C. Mawas, and J. Gerdes, for generous gifts of monoclonal antibodies used in this study, Dr. P. Lane for the generous gift of recombinant human CD40, and Dr. R. Armitage for kindly providing us with recombinant human CD40L.

This work is supported by a programme grant from the British Medical Research Council.

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Received for publication 12 August 1994 and in revised form 28 November 1994.

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