SIMULTANEOUS FLOW CYTOMETRIC ANALYSIS OF HUMAN T CELL ACTIVATION ANTIGEN EXPRESSION AND DNA CONTENT

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The process of stimulating small, resting T lymphocytes to proliferate is accompanied by the appearance of new cell surface antigens. These antigens, characterized as T cell activation antigens by their presence on activated T cells and their absence from resting T cells, are not necessarily T cell specific. Some, like the transferrin receptor (1–6), the insulin receptor (7), and the 4F2 antigen (8), have a broad cellular distribution and are present on proliferating cells of many types. The HLA-DR antigen, which appears on T cells after activation (9–13), is not restricted to cells of the immune system but nevertheless plays a critical role in the regulation of the immune response. At present, only one T cell activation antigen, Tac (14), has been found solely on activated T cells.

We have used monoclonal antibodies (mAb)[1] against seven different molecularly defined T cell activation antigens to explore the order of events following mitogen-dependent activation. Immunofluorescence in combination with the simultaneous analysis of DNA content has allowed the classification of activation antigens according to the kinetics of their appearance and the degree to which they are associated with proliferating cells.

Materials and Methods

MAb. MAb OKT11A, OKT10 (4, 15), and OKT9 (3, 6) were the gift of Dr. Gideon Goldstein (Ortho Pharmaceutical, Raritan, NJ). MAb 5E9 (3) and 4F2 (8) were from Dr. Bart Haynes (Duke University, Durham, NC). MAb B1 49.9 (49.9) and B1 192 (19.2) were the gifts of Dr. Bernard Malissen and Dr. Claude Mawas (University of Monseilles, Marseilles, France). The anti-Tac Ab was the generous gift of Dr. Thomas Waldmann (National Institutes of Health, Bethesda, MD). The monoclonal anti-HLA-DR Ab was purchased from Bethesda Research Laboratories (Rockville, MD). OKT11A recognizes 100% of thymocytes and 100% of peripheral blood lymphocytes that rosette with sheep erythrocytes and is thought to be directed against the E rosette receptor on lymphocytes. The other Ab recognize cell surface proteins that

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[1] *Abbreviations used in this paper:* Ab, antibody; E^+, rosetting blood mononuclear cells; E^-, nonrosetting blood mononuclear cells; FITC-RaMIg, fluoresceinated $(Fab')_2$ of Ab to mouse immunoglobulin; HO342, DNA dye Hoechst 33342; IL-2, interleukin 2; mAb, monoclonal antibody; PHA, phytohemagglutinin-P.

appear on T cells after lectin-induced stimulation. 5E9 and OKT9 recognize the transferrin receptor (3, 6). OKT10 recognizes a 46,000-mol wt antigen (3, 15), whereas the anti-Tac Ab recognizes a protein that appears to be restricted to activated T cells (14). Ab 19.2 recognizes a 240,000-dalton component, whereas 49.9 recognizes a 55,000-dalton protein on the surface of activated T cells (16).

Cell Preparation. Peripheral blood mononuclear leukocytes were obtained from normal human volunteers by Ficoll-Hypaque density gradient centrifugation of defibrinated blood. A population enriched for T cells was obtained by rosetting with neuraminidase-treated sheep erythrocytes. The resulting rosetting (E^+) and nonrosetting (E^-) cells were incubated separately in 100-mm plastic petri dishes in 8% fetal calf serum at a concentration of 2–5 × 10^6/ml. The adherent E^- cells were recovered with a rubber policeman after removing nonadherent cells with three media washes. The E^--adherent cells were counted and placed in a 75-cm^2 flask (Falcon Labware, Oxnard, CA) together with the nonadherent E^+ cells in a ratio of 1:25 (E^- adherent/E^+ nonadherent).

Lectin Stimulation. T cell-enriched lymphocytes were placed in bulk culture in 75-cm^2 flasks at a concentration of 1–2 × 10^6/ml in RPMI 1640 medium with penicillin and streptomycin and supplemented with 8% fetal calf serum. Flasks received either 2 μg/ml phytohemagglutinin-P (PHA) (Difco Laboratories, Detroit, MI) and 10 μg/ml conconavalin A (Sigma Chemical Co., St. Louis, MO) or medium alone. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Aliquots containing ~1–2 × 10^7 cells were removed at various times for analysis of cell surface antigens.

Staining Procedures. Cultured cells were washed once in warm medium with special attention given to dispersing clumped cells after both centrifugations. The DNA dye Hoechst 33342 (HO342) (Hoechst AG, Frankfurt, Federal Republic of Germany) was added from a 100-μM solution to 10^6 cells/ml in RPMI 1640 to a final concentration of 5 μM. At 5 μM, HO342 has previously been shown to be a stoichiometric stain for DNA (17, 18). Cells were incubated at 37°C for 45–60 min. The cells were pelleted, washed twice in ice cold medium, and resuspended to a concentration of 10^7 cells/ml in medium with 0.2% soybean trypsin inhibitor as carrier protein. 50 μl of cells (or 5 × 10^5 cells) was added per well to a 96-well round-bottomed microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA) and incubated with either mAb (freshly centrifuged at 100,000 *g* for 30 min) or purified normal mouse IgG. After 1 h on ice, cells were washed three times and incubated for an additional hour with 25 μl of a 1:20 dilution of the fluorescein-conjugated $F(ab')_2$ fragment of rabbit anti-mouse IgG (FITC-RaMIg) (N. L. Cappel Laboratories Inc., Cochranville, PA). Cells were washed four times and maintained at 0–4°C until flow cytometric analysis.

Flow Cytometry. The dual illumination beam instrument has been described (19, 20). Briefly, the blue fluorescence of HO342 was excited by ultraviolet light (350–370 nm) from a mercury arc lamp; the emitted fluorescence was detected by a photomultiplier fitted with a 420-nm long-pass filter and a 450-nm broad-band interference filter. The green fluorescence (530–570 nm) of fluorescein was excited by the 488-nm beam of an argon ion laser. Two-parameter distributions were accumulated using a hardwired analyzer incorporating a storage display oscilloscope. Windows on the display were set such that <3% of the cells stained with FITC-RaMIg alone were positive. Except for the OKT11A staining, which served as a positive control, the percentage of total lectin-stimulated cells staining with a given Ab was determined by subtraction of the percentage of stained cells from cultures that received no lectin. At least 10,000 cells were analyzed for determination of the percentage of immunofluorescence-positive cells as well as their cell cycle position. Pictures represent 1,000 cells (each white dot represents one cell).

Results

Temporal Appearance of Activation Antigens on Mitogen-stimulated T Cells. One potential means of gaining insight into an activation antigen's function is to determine the kinetics of its appearance and to compare this with the acquisition of functional T cell activities. It was therefore of interest to see whether activation antigens could be distinguished and classified on the basis of the rate of appearance after activation of

resting T cells. Peripheral blood T cells, prepared by rosetting with sheep erythrocytes, were stimulated with PHA and examined at various times for their reactivity with each of seven mAbs to previously defined T cell activation antigens.

The results of eight separate experiments, summarized in Fig. 1, showed that each antigen was present initially on <6% of resting T cells. Lymphocyte activation by PHA evoked the appearance of four antigens within the first 24 h. The 4F2 antigen, Tac, the 49.9 antigen, and the transferrin receptor appeared on an average of 21–55% stimulated cells within 24 h. By contrast, both the HLA-DR antigen and the 19.2 antigen did not appear until much later and were not found on >10% of stimulated cells until 72 h. The kinetics of the expression of the OKT10 antigen were intermediate in nature, being found on an average of 12% of cells at 24 h and increased gradually to an average of almost 50% at 96 h. These results indicated that each of the activation antigens had a characteristic and reproducible time of appearance after T cell activation by mitogens.

To investigate whether the four earliest antigens could be further distinguished, an earlier time course was performed. One such experiment is shown in Fig. 2a. The 4F2 antigen was the first antigen to appear, as it was detectable within 4 h of PHA addition (Fig. 2). The 4F2 antigen expression increased continuously and progressively with time both with respect to the number of $4F2^+$ cells and the intensity of 4F2 antigen expression. The transferrin receptor appeared within the first 4–8 h (Fig. 2 b), whereas Tac expression was first distinguishable from background at 8 h, and by 16 h a marked shift in antigen intensity was evident. The 49.9 antigen was marginally detectable at 8 h and became readily apparent at 16 h. Thus, the four antigens that appear earliest upon activation could further be ordered when a finer time course was carried out. Other experiments showed that the absolute times when a particular antigen became accessible varied slightly from experiment to experiment but the relative order remained the same: 4F2 was the earliest, followed by the transferrin receptor, and finally Tac and the 49.9 antigen.

The 4F2 Antigen, Tac, the 49.9 Antigen, and the Transferrin Receptor Appear before DNA

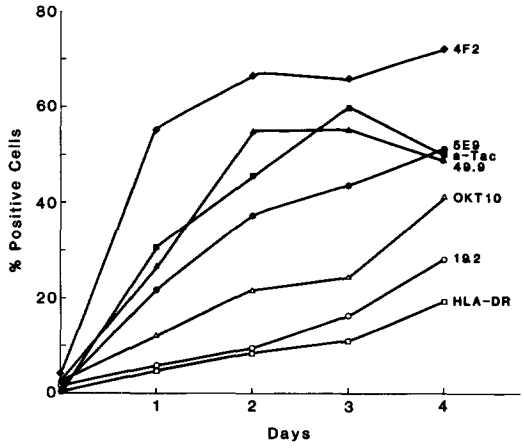

FIG. 1. Time course of appearance of T cell activation antigens. A summary of eight activation experiments shows the average reactivity of seven monoclonal Abs on T cells (E^+) stimulated at 2 × 10^6/ml with 2 μg/ml PHA. 1,000 cells were analyzed per sample and values presented are those after subtracting control values, incubated without PHA for the corresponding times.

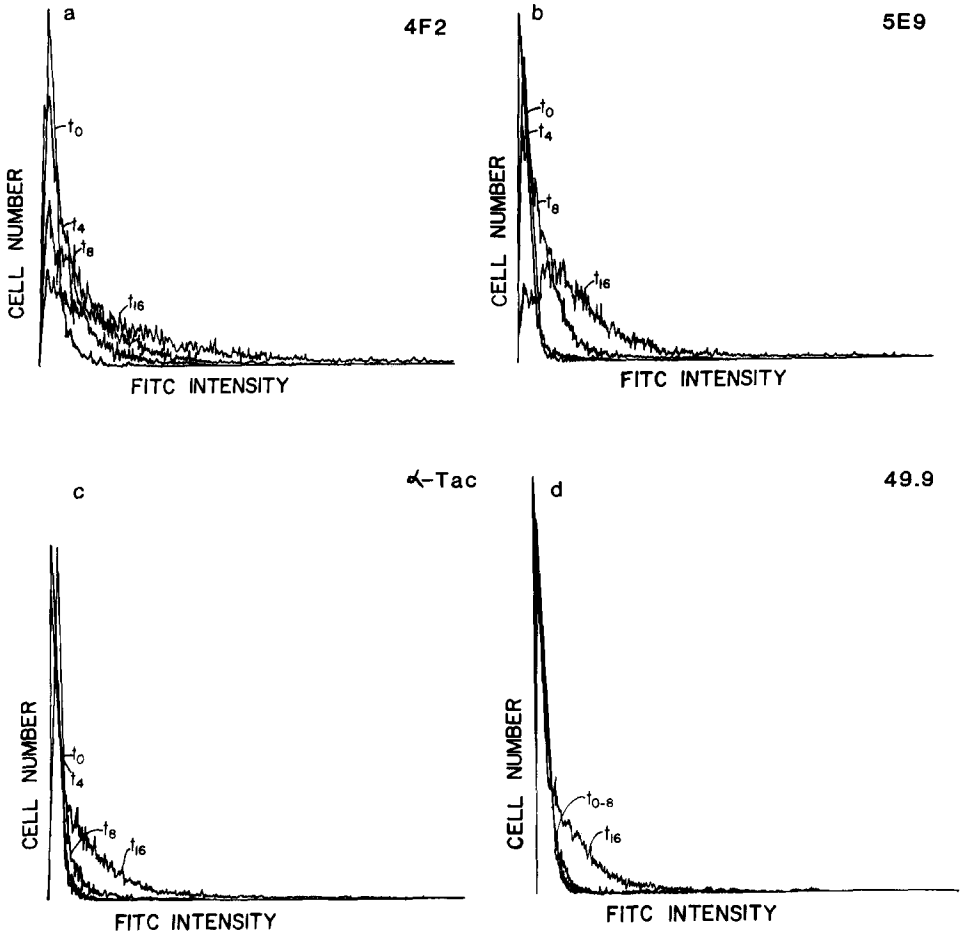

FIG. 2. Temporal appearance of the four earliest-activation antigens. Bulk T cell cultures were initiated as in Fig. 1 at 24, 16, 8, 4, and 0 h before harvesting. Cells were divided into six parts and analyzed for reactivity with (a) 4F2, (b) 5E9 and OKT9, (c) anti-Tac, (d) 49.9, and nmIg and OKT11 (not shown). Reactivity with nmIgG was <3% and reactivity with OKT11 was ~95% during the course of the experiment.

Synthesis. The very early kinetics of the 4F2 antigen, Tac, the 49.9 antigen, and the transferrin receptor seemed to indicate that these antigens appeared on mitogen-stimulated cells before DNA synthesis. To examine this question, we used the dye HO342, which stains DNA stoichiometrically, to analyze individual cells simultaneously for DNA content and immunofluorescence (20). T cells were stimulated with PHA and at various times cells were removed and incubated for 45 min at 37°C with 5 μM HO342 and then cells were reacted with Ab and analyzed by flow cytometry. The results of a typical experiment clearly indicate that all four antigens appear before any increase in DNA content (Figs. 3C–F). The four antigens were found on 20–50% of PHA-treated T cells at 24 h, at which time there was no marked increase in the percentage of cells in the S, G_2, and M cell cycle compartments (1.1%) over the control of cells cultured without PHA for 24 h (1.2%).

A summary of nine experiments in which cells were analyzed for antigen expression

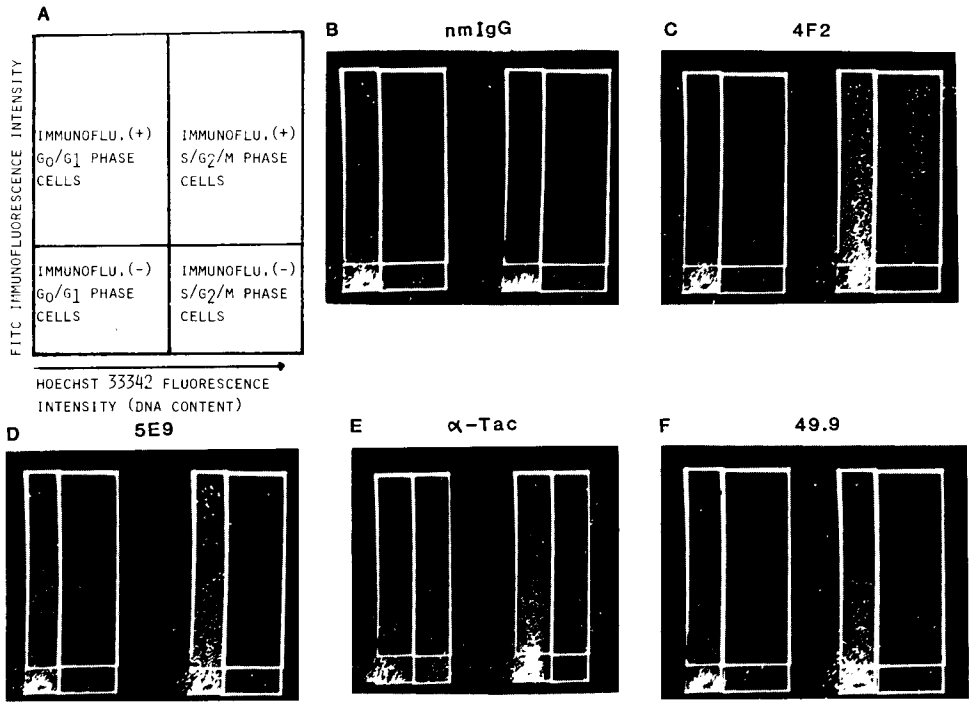

FIG. 3. Four T cell activation antigens appear before DNA synthesis. T cells were stimulated with PHA as before and analyzed simultaneously for immunofluorescence and DNA content at 24 h. (A) Histogram key. Cells were stained with (B) nmIgG, (C) 4F2, (D) 5E9, (E) a-Tac, and (F) 49.9. Cells appearing in the S, G_2, and M window in the right panel of C are clumped (see text). Immunofluorescent windows are fixed such that reactivity with FITC-RaMIg was <3% and windows for DNA content were set by comparison with Hoechst fluorescence patterns of either mitogen-stimulated cultures 72 h or older, or long-term IL-2 T cells of normal karyotype.

and DNA content after activation is shown in Fig. 4. When analyzed at 24 h, the transferrin receptor, the 4F2 antigen, the 49.9 antigen, and Tac showed a net increase of from 22 to 53%, whereas the percentage of cells in S, G_2, or M stages of the cell cycle had increased <1%. Because these four antigens appeared before any cellular proliferation, it is clear that the increase in antigen expression occurred in cells having undetectable amounts of these antigens at the cell surface. The increase in antigen expression was not due to the expansion of a small percentage of $4F2^+$, Tac^+, 49.9^+, and transferrin receptor-bearing cells present before activation. This result is in confirmation of previous results reported for Tac (14) and the 4F2 antigen (8). The term "early" proteins is used to describe those viral proteins in virus-infected cells that are made before replication of viral DNA. We have adopted this terminology to the T cell activation antigens and will refer to the 4F2 antigen, Tac, the 49.9 antigen, and the transferrin receptor as early antigens.

Growth Association of the Early Antigens. Because Tac, the 4F2 antigen, the 49.9 antigen, and the transferrin receptor appeared before DNA synthesis, we next investigated the possibility that one of these markers could be used to predict later cellular events such as DNA synthetic capacity and cellular proliferation. Therefore, we examined that fraction of cells appearing in the proliferative phases of the cell

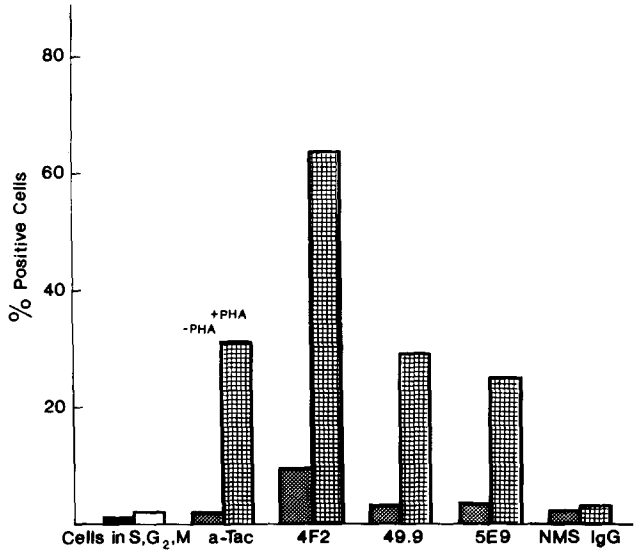

FIG. 4. The transferrin receptor, Tac, the 4F2 antigen, and the 49.9 antigen are early T cell activation antigens. The histogram summarizes nine separate experiments at 24 h. The background immunofluorescence staining was analyzed on resting T cells (no PHA) (stippled bars) and the highest background (10%) was associated with the 4F2 mAb. DNA synthesis had not begun at the time of staining, there was only ~1% more S, G_2, or M phase cells in the PHA-stimulated cultures (clear bar vs. darkened bar). However, a striking increase in expression of all four early antigens was demonstrated by PHA-stimulated T cells (cross-hatched bars).

cycle (i.e., S, G_2, or M) that expressed these early-activation antigens. When PHA-stimulated cells were examined at day 3—a time when a large fraction of the cells were in S, G_2, or M—a striking result was obtained. Over 90% of the activated T cells that were in S, G_2, or M were expressing each of the four early-activation antigens (Table 1, Fig. 5 B–E). Representative pictures demonstrate that 72 h after the addition of PHA there is a dramatic shift in both expression of the early-activation antigens (vertical axis) and the number of cells in the proliferative compartments of the cell cycle (horizontal axis). By focusing on just those cells in S, G_2, or M (those to the right of the medial vertical line), it is apparent that although they fail to react with normal mouse IgG (Fig. 5 A, right panel), >95% manifest a strong reactivity with 4F2 and Tac (right panels of Fig. 5 B and E) and 90% react with 49.9 and antibody to the transferrin receptor (right panels of Fig. 5 C and D). Because virtually every cell found in the S, G_2, or M phase of the cell cycle expresses each of the early-activation antigens, it indicates that before DNA synthesis occurs in T cells they will express each of these antigens.

The degree to which cells in S, G_2, or M express Tac and the 49.9 antigen, which to date have only been found on activated T cells, depends on the purity of T cells in the culture. It is possible that the 5–10% subpopulation of cells in S, G_2, or M that fail to express these antigens represent contamination by proliferating non-T cells. A second difficulty that must be overcome is clumping of cells. Cells clumped as doublets will appear in the S, G_2, or M window at the tetraploid (4C) position. Clumped cells can be monitored and distinguished in three ways. First, clumping is random and appears in triplets, and quadruplicates will also appear at the 6C and 8C positions.

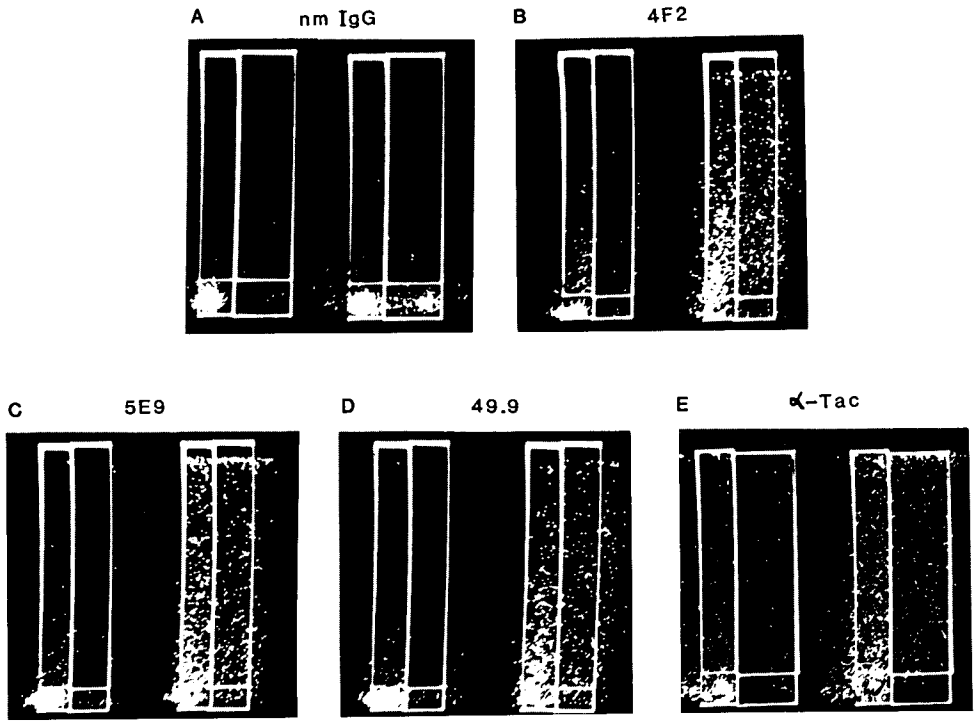

FIG. 5. The expression of the four early T cell activation antigens on cells in the proliferative phase of the cell cycle. T cells were stimulated with PHA as before and analyzed at 72 h as in Fig. 3. Pictures were chosen to most closely approximate average values (see Table I). (A) nmIgG, (B) 4F2, (C) 5E9, (D) 49.9, (E) α-Tac. S, G_2, and M phase cells are to the right of the central vertical white line, and the majority of cycling cells are immunofluorescence positive (above horizontal line). Many G_0/G_1 phase cells (left of vertical bar) are also immunofluorescence positive.

Second, DNA synthesis is continuous and cells in the S phase produce a continuous pattern. In cultures exhibiting zero to moderate proliferation, cells clumped as doublets appear discontinuous, with a disproportionately low fraction of cells intermediate between the diploid and tetraploid clusters. Finally, multiple samples are always analyzed from the same culture and contain the same proportion of cells in the S, G_2, or M window so that the presence of clumps in any one sample is discernible.

T Cell Proliferation Is Initially Independent of the Expression of HLA-DR, T10, and the 19.2 Antigens. There are several reports that the appearance of HLA-DR is a relatively late occurrence after mitogen-dependent activation of T cells (11, 13, 21). However, it remains uncertain whether T cells must express HLA-DR before proliferating. Hence, it was of interest to determine what percentage of proliferating cells bore HLA-DR. Cellular proliferation was measured by estimating the DNA content of individual cells and determining the percentage of cells in the S, G_2, and M phases of the cell cycle. Only populations having at least 10% of the cells in S, G_2, and M were analyzed further for HLA-DR expression. As shown in Table I, when only those cells that had entered the proliferative phase of the cell cycle were analyzed, only ~20% of these were HLA-DR^+. Because we have seen no cell cycle-specific changes in HLA-DR expression (data not shown), we conclude that the ability of T cells to proliferate in a mitogen-dependent system is apparently independent of T cell HLA-DR expres-

TABLE I

Expression of Activation Antigens on Dividing Cells

Ab	Number of experiments	Percent immunofluorescent cells in S, G_2, and M	Range
4F2	12	94.8 ± 5.2	83–100
5E9	13	89.6 ± 7.9	79–100
499	9	90.1 ± 4.8	80–97
α-Tac	8	95.9 ± 4.6	90–100
HLA-DR	11	18.3 ± 12.0	3–37
T10	8	54.5 ± 20.2	24–83
192	6	33.4 ± 11.1	17–47

sion. Similar conclusions have been reached by others (22) who have failed to inhibit mitogen-dependent T cell proliferation with Abs to HLA-DR. Similarly, there is a lack of association between proliferating cells and the expression of the T10 or the 19.2 antigens, as T10 was found on 55% and 19.2 on 33% of cells in S, G_2, or M stages of the cell cycle. Thus, both HLA-DR and antigen 19.2 are expressed on the average after DNA synthesis and may be classified as "late" antigens, whereas the T10 antigen is intermediate both in its temporal appearance and the degree to which it is associated with dividing cells. Although expression of these late antigens is not a prerequisite of cellular proliferation for the majority of T cells, our data do not permit us to rule out the possibility that their expression is necessary for the proliferation of a subpopulation of T cells.

Discussion

This study demonstrates that of the seven T cell activation antigens examined here, four are expressed at the surface of activated T cells before the onset of DNA synthesis. These four proteins, called early-activation antigens, were the transferrin receptor, the T cell activation antigen Tac, and two antigens recognized by the mAb 4F2 and 49.9. This finding is similar to the previously reported early expression of insulin-binding sites on activated lymphocytes (7) and confirms the result of Uchiyama et al. (14) that Tac is expressed before DNA synthesis. A second, unanticipated result was that these same four early antigens are found on virtually all T cells by the time they reach the S phase of the cell cycle. Thus, when just the cells in the S, G_2, or M stages of the cell cycle were examined, an average of 90–96% of these cells expressed each of the four early antigens. The fact that essentially all S, G_2, or M cells were $4F2^+$, 49.9^+, Tac^+, or $5E9/OKT9^+$ was not due to an overlap from the increased HO342 fluorescence because (*a*) the effect seen was specific for the early antigens and was not seen for the three later-appearing antigens (see Table I) and (*b*) excitation and emission of HO342 and FITC occurred independently as the cell passed two separate beams, (*c*) immunofluorescence results were unchanged when HO342 was deleted from the staining protocol (not shown).

The fact that each early activation antigen is expressed before the onset of DNA synthesis suggests that subsequent cell cycle progression does not occur before the function of each of these early antigens has been executed. Although a cell-surface protein whose function was required for T cell growth would be expected to appear on all T cells before their entering the proliferative phase of the cell cycle, our data do

not establish an obligatory role for these proteins in promoting cell growth. However, the fact that these antigens are induced to appear on activated T cells and are present on essentially all T cells before the initiation of DNA synthesis marks these proteins as excellent candidates for molecules causally related to cell growth. One general approach that might establish a causal link between expression of the early activation antigens and subsequent events in the cell cycle is to see what the effect of inhibiting the function of the activation antigen has on cell growth. Abrogation of the antigen function can be achieved by blocking with antibody to the antigen, or in the case of an antigen that is a known receptor, by depriving the appropriate ligand. Recently there have been reports that Ab to each of the four early-activation antigens reproducibly but not completely block T cell proliferation (5, 23), whereas OKT10, AB19.2, and Ab to HLA-DR do not (T. Cotner, unpublished observations; 22). The failure to achieve complete inhibition may result from the fact that a given mAb sees such a restricted site on its target antigen that it may not completely compromise the function of that molecule. For instance, four mAb to the transferrin receptor (5E9, OKT9, B3/25, CP200) fail to block the binding of transferrin (3, 4; T. Cotner, unpublished observations), and the only instance in which a mAb successfully blocks transferrin binding was achieved by specifically selecting Ab for that property (24).

It is interesting to correlate what is known about the role of the early activation antigens with what is known about the growth-promoting activity of their ligands. Sato and his co-workers (reviewed in 25) have defined serum-free media for a number of cell lines and this has led to the conclusion that transferrin (or lactoferrin) is a universal requirement for the long-term growth of all mammalian cell types. The requirement for insulin, whose receptor is an early-activation antigen (7), is not as stringent, but it, too, is a component of all defined media. Of special interest is the fact that both transferrin and insulin are components of defined media that support the growth of B cells (26) and T cells responding in mixed leukocyte reactions (27, 28). Finally, there is now a considerable body of evidence that interleukin 2 (IL-2) is required to support T cell proliferation (29, 30; for reviews see 31, 32). The IL-2 receptor itself is an activation antigen that appears rapidly (4–8 h [33, 34] after mitogen activation) and would be expected to have the same growth-association property exhibited by the transferrin receptor and the three other early-activation antigens studied here. In examining the cellular distributions of the IL-2 receptor and the early-activation antigens, it was apparent that both Tac and the 49.9 antigen, but not the 4F2 antigen, had cell distributions congruent with that of the IL-2 receptor, i.e., present on all IL-2-dependent cells and lines but absent from T and B lymphoblastoid cell lines. The IL-2 receptor had previously been shown to be present on three lines derived from cutaneous T cell lymphomas (35) that are not dependent on exogenous IL-2. We have examined two of these, HUT-78 and HUT-102, and both Tac and the 49.9 antigen are strongly expressed on HUT-102 and detectable on HUT-78. Recently, we have learned that at least a Tac recognizes the IL-2 receptor because it competes with IL-2 for binding to IL-2-dependent cells (36). The supposition that there is a causal link between expression of the early activation antigens and cell growth is based on their absolute expression on growing cells and the ability of Ab to these antigens to inhibit, albeit incompletely, T cell proliferation. Until Ab are produced that are more effective in blocking T cell growth, this hypothesis relies heavily on the previously demonstrated growth-promoting activity of their ligands,

transferrin, IL-2, and insulin. Regardless of what the functional activities of the remaining early-activation antigens turn out to be, it seems reasonable to speculate that their functions will be important in influencing G_1 transit and the stimulation of DNA synthesis.

Summary

Cell-surface antigens that are induced to appear on T cells activated by the lectin phytohemagglutinin-P (PHA) can be classified both on the basis of the kinetics of their appearance and on their growth-association properties. Seven distinct T cell activation antigens, defined by monoclonal antibodies, were classified as early, intermediate, or late antigens based on their temporal appearance relative to DNA synthesis. Four antigens, the transferrin receptor, the T cell activation antigen Tac, the 4F2 antigen, and the 49.9 antigen were early antigens, whereas the OKT10 antigen appeared at intermediate times and both HLA-DR and antigen 19.2 appeared late. The use of a dye, Hoechst 33342, which stains DNA stoichiometrically, allowed the simultaneous analysis of immunofluorescence and cell cycle position of individual cells. This analysis unexpectedly revealed that essentially all cells in the proliferative phase of the cell cycle expressed each of the four early-activation antigens. The correlation between expression of the four early-activation antigens and T cell proliferation suggests that these molecules are important for the growth of all T cells. The relationship of two of these activation antigens, known to be the receptors for transferrin and interleukin 2, a T cell growth factor, is discussed with special reference to the roles of their ligands in supporting the growth of T cells.

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References

1. Omary, M. B., I. S. Trowbridge, and J. M. Minowada. 1980. Human cell-surface glycoprotein with unusual properties. *Nature (Lond.).* **286:**888.
2. Judd, W., C. A. Poodry, and J. L. Strominger. 1980. Novel surface antigen expressed on dividing cells but absent from nondividing cells. *J. Exp. Med.* **152:**1430.
3. Haynes, B. F., M. Hemler, T. Cotner, D. L. Mann, G. S. Eisenbarth, J. L. Strominger, and A. S. Fauci. 1981. Characterization of a monoclonal antibody (5E9) that defines a human cell surface antigen of cell activation. *J. Immunol.* **127:**347.
4. Terhorst, C., A. van Agthoven, K. Leclair, P. Snow, E. Reinherz, and S. Schlossman. 1981. Biochemical studies of the human thymic differentiation antigens T6, T9, and T10. *Cell.* **23:**771.
5. Trowbridge, I. S., and M. B. Omary. 1981. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc. Natl. Acad. Sci. USA.* **78:**3039.
6. R. Sutherland, D. Delia, C. Schneider, R. Newman, J. Kemshead, and M. Greaves. 1981. Ubiquitous cell-surface glycoprotein on tumor cells is proliferation-associated receptor for transferrin. *Proc. Natl. Acad. Sci. USA.* **78:**4514.
7. Helderman, J. H., and T. B. Strom. 1979. Role of protein and RNA synthesis in the development of insulin binding sites on activated thymus-derived lymphocytes. *J. Biol. Chem.* **254:**7203.

8. Haynes, B. F., M. E. Hemler, D. L. Mann, G. S. Eisenbarth, J. Shelhamer, H. S. Mostowski, C. A. Thomas, J. L. Strominger, and A. S. Fauci. 1981. Characterization of a monoclonal antibody (4F2) that binds to human monocytes and to a subset of activated lymphocytes. *J. Immunol.* **126:**1409.
9. Evans, R. L., T. J. Faldetta, R. E. Humphreys, D. M. Pratt, E. J. Yunis, and S. F. Schlossman. 1978. Peripheral human T cells sensitized in mixed leukocyte culture synthesize and express Ia-like antigens. *J. Exp. Med.* **148:**1440.
10. Suciu-Foca, N., E. Susinno, P., McKiernon, C. Rohowsky, J. Weiner, and P. Rubinstein. 1978. DRw determinants on human T cells primed against allogeneic lymphocytes. *Transplant. Proc.* **10:**845.
11. DeWolf, W. C., S. F. Schlossman, and E. J. Yunis. 1979. DRw antisera react with activated T cells. *J. Immunol.* **122:**1780.
12. Greaves, M. F., W. Verbi, H. Festenstein, C. Papesteriades, D. Jeroquimada, and A. Hayward. 1979. "Ia-like" antigens on human T cells. *Eur. J. Immunol.* **9:**356.
13. Ko, H., S. M. Fu, R. J. Winchester, D. T. Y. Yu, and H. G. Kunkel. 1979. Ia determinants on stimulated human T lymphocytes. Occurrence on mitogen and antigen-activated T cells. *J. Exp. Med.* **150:**246.
14. Uchiyama, T., S. Broder, and T. A. Waldmann. 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. *J. Immunol.* **126:**1393.
15. Cotner, T., H. Mashimo, P. C. Kung, G. Goldstein, and J. L. Strominger. 1981. Human T cell surface antigens bearing a structural relationship to HLA antigens. *Proc. Natl. Acad. Sci. USA.* **78:**3858.
16. Cotner, T., M. Hemler, and J. L. Strominger. 1981. Human T cell proteins recognized by rabbit heteroantisera and monoclonal antibodies. *Int. J. Immunopharmacol.* **3:**255.
17. Arndt-Jovin, D. J., and T. M. Jovin. 1977. Analysis and sorting of living cells according to deoxyribonucleic acid content. *J. Histochem. Cytochem.* **25:**585.
18. Loken, M. R. 1980. Simultaneous quantitation of Hoechst 33342 and immunofluorescence on viable cells using a fluorescence activated cell sorter. *Cytometry.* **1:**136.
19. Shapiro, H. M. 1981. Flow cytometric estimation of DNA and RNA content in intact cells stained with Hoechst 33342 and Pyronin Y. *Cytometry.* **2:**143.
20. Williams, J. M., H. M. Shapiro, E. L. Milford, and T. B. Strom. 1982. Multiparameter flow cytometric analysis of lymphocyte subpopulation activation in lectin stimulated cultures. *J. Immunol.* **128:**2676.
21. Hercend, T., J. Ritz, S. F. Schlossman, and E. L. Reinherz. 1981. Comparative expression of T9, T10, and Ia antigens on activated human T cell subsets. *Hum. Immunol.* **3:**247.
22. Palacios, R. 1982. Concanavalin A triggers T lymphocytes by directly interacting with their receptors for activation. *J. Immunol.* **128:**337.
23. Cotner, T., J. M. Williams, T. B. Strom, and J. L. Strominger. 1983. The relationship between early T cell activation antigens and T cell proliferation. Immunopharmacology: 2nd International Symposium. J. E. Hadden, editor. Pergamon Press, London. In press.
24. Trowbridge, I. S., and F. Lopez. 1982. Monoclonal antibody to the transferrin receptor blocks transferrin binding and inhibits human tumor cell growth *in vitro. Proc. Natl. Acad. Sci. USA.* **79:**1175.
25. Barnes, D., and G. Sato. 1980. Growth of cultured cells in serum-free medium. *Anal. Biochem.* **102:**255.
26. Iscove, N. N., and F. Melchers. 1978. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. *J. Exp. Med.* **147:**923.
27. Snow, E. C., T. L. Feldbush, and J. A. Oaks. 1981. The effect of growth hormone and insulin upon mixed leukocyte culture responses and the generation of cytotoxic T lymphocytes. *J. Immunol.* **126:**161.

28. Strom, T. B., and J. D. Bangs. 1982. Human serum-free mixed lymphocyte response: the stereospecific effect of insulin and its potentiation by transferrin. *J. Immunol.* **128:**1555.
29. Morgan, D. A., F. W. Ruscetti, and R. Gallo. 1976. Selective *in vitro* growth of T-lymphocytes from normal human bone marrow. *Science (Wash. DC).* **193:**1007.
30. Gillis, S., and K. A. Smith. 1977. Long term culture of tumor-specific cytotoxic T cells. *Nature (Lond.).* **268:**154.
31. Smith, K. A. 1980. T cell growth factor. *Immunol. Rev.* **51:**337.
32. Ruscetti, F. W., and R. C. Gallo. 1981. Human T lymphocyte growth factor: regulation of growth and function of lymphocytes. *Blood.* **57:**379.
33. Larsson, E.-L., and A. Coutinho. 1979. The role of mitogenic lectins in T-cell triggering. *Nature (Lond.).* **280:**239.
34. Stadler, B. M., S. F. Dougherty, J. J. Farrar, and J. J. Oppenheim. 1982. Relationship of cell cycle to recovery of IL2 activity from human mononuclear cells, human and mouse T cell lines. *J. Immunol.* **127:**1936.
35. Gootenberg, J. E., F. W. Ruscetti, J. W. Meir, A. Gazdar, and R. C. Gallo. 1981. Human cutaneous T cell lymphoma and leukemia cell lines produce and respond to T cell growth factor. *J. Exp. Med.* **154:**1403.
36. Leonard, W. J., J. M. Depper, T. Uchiyama, T. A. Waldmann, and W. C. Greene. 1982. A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor. *Nature (Lond.).* **300:**267.