Markers for Diagnosis and Monitoring of Human Cancer

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CONTRIBUTORS

R. ACCINNI Istituto di Ricerche Cardiovascolari "G. Sisini", Università degli Studi di Milano, Via F. Storza, 35-20122 Milan, Italy
R. S. ACCOLLA Ludwig Institute for Cancer Research, 1066 Epalinges, Lausanne, Switzerland
A. ALBERTINI Istituto di Chimica della Facoltà Medica di Brescia, Brescia, Italy
K. D. BAGSHAWE Charing Cross Hospital Medical School, Fulham Palace Road, London W6 8RF, UK
M. A. BAILO Istituto di Ricerche Cardiovascolari "G. Sisini", Università degli Studi di Milano, Via F. Storza, 35-20122 Milan, Italy
A. BARGELLESI Ludwig Institute for Cancer Research, Epalinges, Switzerland
A. BARTORELLI Istituto di Immunologia Clinica, Università degli Studi di Milano, Via F. Storza, 35-20122 Milan, Italy
L. BASCHIERI Cattedra di Patologia Medica II°, Università di Pisa, Via Roma 67, 56100 Pisa, Italy
C. BIANCARDI Istituto di Ricerche Cardiovascolari "G. Sisini", Università degli Studi di Milano, Via F. Storza, 35-20122 Milan, Italy
E. BOMBARDIERI Department of Nuclear Medicine, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via G. Venezian 1, 20133 Milan, Italy
G. BONORA I° Clinica Medica dell'Università di Bologna, Policlinico S. Orsola, Via Massarenti 9, Bologna, Italy
F. BUCHEGGER Institute of Biochemistry, University of Lausanne, 1066 Epalinges, Lausanne, Switzerland
P. BURTIN Institut de Recherches Scientifique sur le Cancer, Villejuif, France
B. BUSNARDO Istituto di Semeiotica Medica, University of Padua, Padua, Italy
N. A. CARPENTIER WHO Immunology Research and Training Centre, Hematological Division, Department of Medicine and Department of Surgery, University of Geneva, 1211 Geneva, Switzerland
S. CARREL Ludwig Institute for Cancer Research, 1066 Epalinges, Lausanne, Switzerland
V. CAVALCA Istituto di Ricerche Cardiovascolari "G. Sisini", Università degli Studi di Milano, Via F. Storza, 35-20122 Milan, Italy

M. CECCHETTIN Istituto di Chimica della Facoltà Medicà di Brescia, Brescia, Italy
P. CHOLLET WHO Immunology Research and Training Centre, Division of Oncohematology, Department of Medicine and Department of Surgery, University of Geneva, 1211 Geneva, Switzerland
J. COLLETTE Laboratory of Radioimmunology, University of Liège, Liège 1, Belgium
G. CORTE Institutes of Biochemistry and Microbiology, University of Genoa, Genoa, Italy
D. CUPPISSOL Laboratoire d'Immunopharmacologie des Tumeurs, INSERM U-236, ERA-CNRS No 844, Centre Paul Lamarque, B.P. 5054 34 033, Montpellier, Cédex, France
E. DINSDALE Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey SM2 5PX, UK
T. S. EDGINGTON Department of Molecular Immunology, Scripps Clinic and Research Foundation, 10666 North Pines Road, La Jolla, California 92037, USA
R. EGELI WHO Immunology Research and Training Centre, Hematological Division, Department of Medicine and Department of Surgery, University of Geneva, 1211 Geneva, Switzerland
C. FAVIER Laboratoire d'Immunopharmacologie des Tumeurs, INSERM U-236, ERA-CNRS No 844, Centre Paul Lamarque, B.P. 5054 34 033, Montpellier, Cédex, France
F. FAVIER Laboratoire d'Immunopharmacologie des Tumeurs, INSERUM U-236, ERA-CNRS No 844, Centre Paul Lamarque, B. P. 5054 34 033, Montpellier, Cédex, France
R. FERRARA Istituto di Ricerche Cardiovascolari "G. Sisini", Università degli Studi di Milano, Via F. Storza, 35-20122 Milan, Italy
G. L. FERRI I° Clinica Medica dell'Università di Bologna, Policlinico S. Orsola, Via Massarenti 9, Bologna, Italy
M. FORNI Clinique Médicale, Department of Medicine, University Hospital of Geneva, 1211 Geneva, Switzerland
P. FRANCHIMONT Laboratory of Radioimmunology, University of Liège, Liège 1, Belgium
F. GELDER Louisiana State University, Shreveport, Louisiana 71130, USA
C. GIANI Cattedra di Patologia Medica II°, Università di Pisa, Via Roma 67, 56100 Pisa, Italy
C. GIRARDET Institute of Biochemistry, University of Lausanne, 1066 Epalinges, Lausanne, Switzerland
M. E. GIRELLI Istituto di Semeiotica Medica, University of Padua, Padua, Italy
D. M. GOLDENBERG Division of Experimental Pathology, Department of Pathology, University of Kentucky Medical Center, Lexington, Kentucky, USA
R. B. HERBERMAN Laboratory of Immunodiagnosis, Building 10, Room 8B02, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205, USA
J. C. HENDRICK Laboratory of Radioimmunology, University of Liège, Leige 1, Belgium
R. HUNTER Emory University, Atlanta, Georgia 30322, USA
G. LABO I° Clinica Medica dell'Università di Bologna, Policlinico S. Orsola, Via Massarenti 9, Bologna, Italy

P. H. LAMBERT WHO Immunology Research and Training Centre, Hematological Division, Department of Medicine and Department of Surgery, University of Geneva, 1211 Geneva, Switzerland
K. O. LLOYD Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10021, USA
J.-P. MACH Ludwig Institute for Cancer Research, 1066 Epalinges, Lausanne, Switzerland
S. MARIOTTI Cattedra di Patologia Medica II°, Università di Pisa, Via Roma 67, 56100 Pisa, Italy
P. MAURICE WHO Immunology Research and Training Centre, Division of Oncohematology, Department of Medicine and Department of Surgery, University of Geneva, 1211 Geneva, Switzerland
R. A. J. McILHINNEY Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey SM2 5PX, UK
K. R. McINTIRE Diagnosis Branch, Division of Cancer Biology and Diagnosis, Building 31, Room 3A10 National Cancer Institute, Bethesda, Maryland 20205, USA
W. B. MILLER Clinical Surgery, University Medical School, Edinburgh, UK
T. MING CHU Roswell Park Memorial Institute, Buffalo, New York 14263, USA
M. C. MINGARI Ludwig Institute for Cancer Research, 1066 Epalinges, Lausanne, Switzerland
P. MONAGHAN Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey SM2 5PX, UK
A. MORETTA Ludwig Institute for Cancer Research, 1066 Epalinges, Lausanne, Switzerland
L. MORRETTA Ludwig Institute for Cancer Research, 1066 Epalinges, Lausanne, Switzerland
V. MOSHAKIS Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey SM2 5PX, UK
A. R. MOOSSA University of Chicago, Illinois 60637, USA
R. M. NAKAMURA Department of Molecular Immunology, Scripps Clinic and Research Foundation, 10666 North Pines Road, La Jolla, California 92037, USA
A. M. NEVILLE Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey SM2 5PX, UK
Y. NIITSU Department of Internal Medicine, Sapporo Medical College, S-1, W16, Chuoku, Sapporo, 060 Japan
F. PACINI Cattedra di Patologia Medica II°, Università di Pisa, Via Roma 67, 56100 Pisa, Italy
S. PILOTTI Department of Pathology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via G. Venezian 1, 20133 Milan, Italy
A. PINCHERA Cattedra di Medicina Costituzionale e Endocrinologia, University of Padua, Padua, Italy
G. PIZZOCARO Section of Urological Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via G. Venezian 1, 20133 Milan, Italy
J. E. PONTES Urologic Oncology, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, New York 14263, USA
D. RAGHAVAN Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey SM2 5PX, UK
J. G. RATCLIFFE Department of Biochemistry, Royal Infirmary, Glasgow

G4 0SF, UK
J. RITSCHARD Division de Médecine Nucléaire, Department of Medicine, University Hospital of Geneva, 1211 Geneva, Switzerland
B. SERROU Laboratoire d'Immunopharmacologie des Tumeurs, INSERM U-236, ERA-CNRS No 844, Centre Paul Lamarque, B.P. 5054 34 033, Montpellier, Cédex, France
A. SULLIVAN Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey SM2 5PX, UK
D. M. P. THOMSON The Montreal General Hospital, 1650 Cedar Avenue, Montreal, Quebec, Canada H3G 1A4
I. URUSHIZAKI Department of Internal Medicine, Sapporo Medical College, S-1, W16, Chuoku, Sapporo, 060 Japan
U. VERONESI Istituto Nazionale per lo Studio e la Cura dei Tumori, Via G. Venezian 1, 20133 Milan, Italy
P. VEZZADINI I° Clinica Medica dell'Università di Bologna, Policlinico S. Orsola, Via Massarenti 9, Bologna, Italy
P. F. ZANGERLE Laboratory of Radioimmunology, University of Liège, Liège 1, Belgium

PREFACE

The scope of the Serono Symposium held in Milan, Italy, in April 1981, which originated this book, was to stress the interest in identifying reliable and reproducible test systems for early diagnosis and monitoring of tumours of clinical and epidemiological relevance in terms of incidence, severity and curability.

This book is intended to be not an all purpose review of the state of the art, but a selection of lectures and discussions specifically aimed at focalizing the marker problems in their function of early diagnostic and monitoring tools in tumours amenable to a therapeutic approach, i.e. breast, GI tract, lung, urogenital and blood tumours. Methods already described and relevant data are analysed in depth, as well as original approaches opening new lines of development in terms either of methodology or philosophy of research. In addition, basic technical and operational aspects of established or prospective test systems together with their practical implications are considered by renowned experts.

A relevant feature of the book is that a large part is devoted to discussion. Distinguished scientists, selected on the basis of their outstanding clinical or laboratory experience critically discuss the data presented by the lecturers with the aim of reaching a valid conclusion even from the point of view of clinical practice.

We would like to thank all the speakers of the Symposium who made it possible to realize successfully the programme as planned and who rapidly contributed to this book which, therefore, constitutes an up-to-date documentation in the field of tumour marker application.

We are also very grateful to the Serono Symposia for sponsoring this meeting and in particular to Dr S Rossetti and his staff.

April 1981

M. I. COLNAGHI
G. L. BURAGGI
M. GHIONE

CONTENTS

SURFACE MARKERS OF HUMAN LYMPHOCYTES: PHENOTYPIC ANALYSIS OF FUNCTIONALLY DEFINED T CELL SUBPOPULATIONS OR T CELL CLONES*

L. Moretta, G. Corte, A. Moretta, M. C. Mingari and A. Bargellesi

Ludwig Institute for Cancer Research, Lausanne Branch, Epalinges, Switzerland and Institutes of Biochemistry and Microbiology, University of Genoa, Genoa, Italy

Studies of cell surface markers and their ability to define subpopulations of lymphoid cells has been an area of most intensive investigation in human immunology.

This type of research has been further stimulated by the concept that lymphocyte surface markers may be directly involved in functional cell activities and/or interactions among cells. For example, surface immunoglobulin, the principal marker of mature B cells, is directly involved in the antigen induced B cell activation.

With respect to human T cell surface markers, they can be operationally divided into three different groups. (1) Those which define the whole T cell population, such as the receptor for sheep erythrocytes, heteroantisera rendered T specific after serial absorptions with different cell types, and, recently, monoclonal antibodies reported to bind to antigenic determinants uniquely expressed by T cells (Haynes *et al.*, 1980a; Moretta *et al.*, 1981d). (2) Those which are present only on a fraction of T cells, such as antigenic determinants recognized by the TH_1 or TH_2 heteroantisera (Evans *et al.*, 1977, 1978) or by monoclonal

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antibodies, such as the OKT_4, OKT_5 (Reinherz and Schlossman, 1980), 3A1 (Haynes *et al.*, 1980a), 5/9 (Corte *et al.*, 1982), receptors for the FC fragment of different immunoglobulin isotypes (Moretta *et al.*, 1976, 1977), receptors for lectins (Hammaström *et al.*, 1973) and histamine (Saxon *et al.*, 1977). (3) A third group is represented by surface markers expressed only on activated T cells; among these are Ia antigens and a group of antigenic determinants recognized by monoclonal antibodies such as the 4F2 (Eisenbarth *et al.*, 1980) and the MLR 1-4 (Corte *et al.*, 1981).

Most of the presently familiar surface markers do not appear to be directly involved in T cell functions. An important exception is the surface FcR for IgG which is known to be involved in effector functions, such as the antibody dependent cellular cytotoxicity (ADCC) (Perlmann *et al.*, 1976; Perlmann and Cerottini, 1979) and in regulatory functions such as the inhibition of T or B cell proliferation in certain *in vitro* systems (Moretta *et al.*, 1977, 1979; Canonica *et al.*, 1980). FcR for all the different isotypes have been described, however FcR for IgG and IgM have been studied in greater detail. These receptors are virtually absent in thymocytes, whereas they are expressed on distinct sets of peripheral T cells. In addition, Fcγ R are detectable in a large fraction of activated T cells, especially after allogenic stimulation (Moretta *et al.*, 1981a,b).

Recent studies in several laboratories have focused on various activities displayed by isolated T cells bearing FcγR or FcμR (T_G and T_M cells, respectively). For example, in the *in vitro* B cell differentiation induced by pokeweed mitogen (PWM), a strictly T dependent phenomenon, it has been shown that T_G and T_M populations play an antithetical role, T_M cells being the helper cells and T_G the suppressor cells (Moretta *et al.*, 1977). Cells responsible for natural killer (NK) activity and ADCC are restricted to the T_G cell fraction (Pape *et al.*, 1979). T_G cells sharply inhibit the *in vitro* lymphoid colony formation (Canonica *et al.*, 1980). In addition, it has been shown that cells, with the T_G phenotype isolated from the bone marrow of patients with severe aplastic anaemia, suppress the *in vitro* myeloid colony formation in both autologous or allogeneic combinations (Bacigalupo *et al.*, 1980).

Taken together, the above functional analyses indicate that dissection of T cells according to their surface FcR may be useful for restricting some T cell activities to T_G or T_M populations. For example, it appears that naturally-occurring human suppressor cells frequently express surface FcγR (Moretta *et al.*, 1981d). However, identification of functional T cell subsets simply based on the use of FcR is not satisfactory. One of the main problems is the fact that only a small proportion of the cells in a given subset may be involved in the functional activity measured. Obviously, similar difficulties occur when T cell subpopulations are identified by other reagents such as monoclonal antibodies. Thus, the function of individual cells cannot be extrapolated simply by the presence of a given marker.

A more precise correlation between the surface phenotype and a given functional activity of the cell can be achieved by different experimental approaches, namely the combined use of different markers and the analysis of markers present on cloned T cells with defined functional properties.

COMBINED USE OF DIFFERENT MARKERS

The usefulness of this approach is demonstrated by two examples dealing with the phenotypical characterization of the T cells responsible for inducing B cell differentiation (helper T cells) and of the specific cytolytic cells (CTL), respectively.

Surface Phenotype of Helper T Cells

It has been reported that the T cells able to promote the PWM dependent B cell differentiation can be defined by their reactivity with a monoclonal antibody named OKT_4 (Reinherz and Schlossman, 1980). As mentioned above helper cells are also present in the T_M cell fraction. If all of the cells expressing these different phenotypes are indeed helper cells, this would imply that cells responsible for inducing B cell differentiation would represent a large fraction of peripheral T cells, since the antigens defined by OKT_4 and 3A1 monoclonal antibodies or the FcμR are all expressed in over 60% of E rosetting cells. However, recent experimental evidence has indicated that helper cells could be further restricted within a much smaller fraction of peripheral T cells, since all the helper cells appeared to be present, and highly enriched, in a T cell fraction reacting with 5/9 monoclonal antibody (Corte *et al.*, 1982; Moretta *et al.*, 1982). This antibody reacts with only 15-20% of peripheral T cells. In addition, not all of the $5/9^+$ cells express FcμR^+ or $OKT4^+$. Therefore, human cells capable of inducing B cell differentiation can be defined more precisely by the simultaneous detection of different markers such as FcμR, OKT_4, 3A1, 5/9. It should be noted that less than 10% of peripheral T cells simultaneously express all of these markers.

Surface Phenotype of Specific Cytolytic T Cells

As mentioned above, some surface markers are expressed on alloactivated T cells. Among these markers, Ia antigens and FcγR are important in view of their direct involvement in certain immune reactions. FcγR are expressed on 10-15% only of peripheral blood T cells (Moretta *et al.*, 1977), whereas Ia^+ cells represent 1-5% (Greaves *et al.*, 1979). Both of these markers, however, are expressed on high proportions of activated T cells (Moretta *et al.* 1981b; Winchester and Kunkel, 1980). In this context, 4F2 antibody (Eisenbarth *et al.* 1980) binds to activated but not to resting cells. FcγR, Ia and 4F2 antigens have been used for studying the surface phenotype of CTL generated in mixed lymphocyte culture (Moretta *et al.*, 1981b). Presence or absence of Ia antigens on alloactivated T cells did not select for specific CTL. In contrast, fractionation of alloactivated T cells in accordance to either surface FcγR or 4F2 antigens showed that CTL were restricted to the $Fc\gamma R^-$ and to the $4F2^+$ cell populations, respectively. $Fc\gamma R^-$ and $4F2^+$ cell fractions are only partially overlapping as purified $4F2^+$ populations included up to 30% $Fc\gamma R^+$ cells, and $Fc\gamma R^-$ cells were up to 50% $4F2^-$. Further fractionation of

cells based on the combined use of the two markers showed that CTL activity was restricted to the $4F2^+$, $Fc\gamma R^-$ subset. Such cell population usually accounts for about 30% of alloactivated T cells (Moretta *et al.*, 1981b).

ANALYSIS OF SURFACE MARKERS PRESENT IN CLONED T CELLS WITH DEFINED FUNCTIONS

Another experimental approach leading to a precise correlation between cell function and surface phenotype is based on the establishment of cloned T cells with defined functions. T cell clones can be generated under limiting conditions in microculture systems in the presence of "filler" cells and T cell growth factors (TCGF). TCGF, also referred to as "Interleukin 2", has been defined as that material present in culture fluids obtained from lectin, or alloantigen, stimulated lymphocyte cultures which is required for the continuous proliferation of cultured T cells (Smith, 1980).

By using these experimental devices, it is now feasible to analyse the phenotype of T cell clones previously selected for a given function. We have recently used this approach for the phenotypical characterization of T cell clones with different types of cytolytic activities (Moretta *et al.*, 1981e). T cells stimulated in secondary MLC were cloned under limiting conditions in microculture systems using TCGF, derived from PHA stimulated human lymphocytes (Moretta *et al.*, 1981c), and irradiated allogenic cells as filler cells. T cell clones were selected for their cytotoxic capacity against: (1) PHA activated target cells bearing the stimulating alloantigens (CTL activity) (2) L1210 mouse cells coated with rabbit antibody (ADCC); (3) K562 human target cells (NK activity). Cells from microcultures with lytic activity restricted to only one of the target cell types used were expanded in macrowells, employing the culture conditions used for cloning and analysed for different surface markers including rosette formation with sheep erythrocytes (E rosettes), receptors for the Fc portion of IgG or IgM ($Fc\gamma R$ and $Fc\mu R$), and a group of antigens recognized by monoclonal antibodies including Ia, 4F2, OKT_8 and OKT_4 (Table I). All clones were E rosette positive with 73-94% of the cells in individual clones forming rosettes. In 12 out of 14 cytolytic clones, $Fc\gamma R$ were virtually absent, whereas 30-40% of the cells in two clones with ADCC activity were $Fc\gamma R^+$. The percentage of $Fc\mu R^+$ cells in all cytolytic clones was less than 10% but reached 26 and 50% respectively in two of the non-cytolytic clones. Flow cytofluorometric analysis showed that all (cytolytic and non-cytolytic) clones expressed relatively large amounts of Ia antigens. In addition, virtually 100% of the cells in individual clones were Ia^+. Comparable results were obtained when the clones were analysed for the expression of 4F2 antigen. In contrast, only four of the cytolytic clones were $OKT8^+$. Among these clones, three were cytolytic against K562 target cells, whereas only one had CTL activity. Of the seven $OKT8^-$ CTL clones, three were $OKT4^+$. The latter antigen was also expressed in two clones active in ADCC and two (out of four) non-cytolytic clones. Flow cytofluorometric analysis of individual CTL clones indicated that virtually 100% of the cells of a clone were positive or negative for OKT_4 or OKT_8 antigens.

Two clones selected for their high CTL activity were subcloned by limiting

Table I. Surface markers of human T cell clones with different cytolytic activities.

Clone number	Cytolytic activity	SRBC	FcγR	FcμR	Ia	4F2	OKT_8	OKT_4
1	NK (86)[a]	89[b]	1	0	+[c]	+[c]	+[c]	−[c]
2	NK (46)	78	0	8	+	+	−	−
3	NK (75)	75	0	10	+	+	+	−
4	NK (80)	84	1	3	+	+	+	−
5	ADCC (35)	80	42	1	+	+	−	+
6	ADCC (41)	81	29	0	+	+	−	+
7	CTL (39)	93	2	1	+	+	−	+
8	CTL (82)	92	4	3	+	+	+	−
9	CTL (88)	94	0	1	+	ND	−	ND
10	CTL (51)	74	3	10	+	+	−	+
11	CTL (35)	83	0	4	+	+	−	−
12	CTL (27)	73	0	8	+	+	−	−
13	CTL (26)	75	0	8	+	+	−	−
14	CTL (60)	94	0	1	+	+	−	+
15	-	93	1	4	+	+	−	−
16	-	77	0	50	+	+	−	+
17	-	81	1	26	+	+	−	+
18	-	89	2	7	+	+	−	−

[a]Percentage of specific lysis at 30:1 lymphocyte/target ratio. [b]Percentage of rosette forming cells. [c]Presence or absence of surface antigens was analysed by fluorescence activated cell sorter.

dilution. All the subclones have been phenotypically and functionally identical to the parent cell clones suggesting a high degree of phenotypic stability, at least over the time period (2-3) weeks) needed for their isolation.

The fact that noticeable proportions, but not all, of the cells of the expanded clones formed E rosettes (or had detectable FcγR), is likely due to limitation of the techniques used. Alternatively, the possibility exists that cells at different stages of the cell cycle or with different metabolic activity vary in their expression of surface receptors. This possibility is supported by the finding that five individual subclones derived from the E rosette negative fraction of a putative clonal isolate were mostly E rosette positive.

It is of interest that FcγR were present in two clones active in ADCC but without lytic activity against K562 target cells. Conversely, the four clones reactive against K562 target cells were $Fc\gamma R^-$ and devoid of K cell activity. Thus, although ADCC and NK activity are mediated by the same $Kc\gamma R^+$ subset in peripheral blood lymphocytes, they can be dissociated in clones derived from alloreactive MLC populations.

Large amounts of Ia antigens were detected on the surface of all cloned cells analysed, including those with CTL activity. Studies at the population level gave conflicting results as to the expression of Ia antigens on human CTL. Reinherz *et al.*, (1980) reported that CTL activity was restricted to the Ia^- fraction of allo-

activated T cells. Earlier studies from our laboratory indicated that Ia^+ and Ia^- MLC cells exhibited similar levels of specific cytolytic activity. The procedure used in our studies for deriving expanded clones, including the use of TCGF, may favour the proliferation of Ia^+ cells. However, we can conclude that at least a fraction, if not all, of human CTL express Ia antigens. Previous studies on MLC T cell populations have shown that CTL activity was restricted to a FcγR negative subpopulation expressing the 4F2 antigen (Moretta *et al.*, 1981a). Although clonal analysis confirms that CTL are $4F2^+$, it is evident that this antigen cannot be used as a specific marker for CTL, as it is also expressed in non-cytolytic clones. These results are in agreement with our previous studies showing that CTL activity in MLC T cell populations is restricted to the $Fc\gamma R^-$ fraction of the $4F2^+$ populations.

Reinherz *et al.* (1980) have recently reported that CTL generated in MLC express the OKT_8^+, OKT_4^- phenotype. Such phenotype, however, was detectable in only one of eight CTL clones analysed. In addition, three of the OKT_8^- CTL clones expressed the OKT_4 antigen. These results clearly indicate that it is not possible to assign a given function to lymphocytes bearing either the OKT_4 or the OKT_8 antigen. Along this line, it is of note that three of four clones with NK activity were OKT_8^+, OKT_4^-, whereas the two clones active in ADCC expressed the opposite phenotype.

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