INFECTION AND IMMUNITY, May 1982, p. 657–661
0019-9567/82/050657-05$02.00/0

Vol. 36, No. 2

Generation of Cytolytic T-Cell Cultures Displaying Measles Virus Specificity and Human Histocompatibility Leukocyte Antigen Restriction

K. K. SETHI,* I. STROEHMANN, AND H. BRANDIS

Institute of Medical Microbiology and Immunology and Department of Clinical Immunology, Medical Clinic, University of Bonn, Venusberg, 5300 Bonn, Federal Republic of Germany

Received 17 August 1981/Accepted 11 December 1981

In the present study, peripheral blood lymphocytes from eight randomly selected, healthy, measles virus-seropositive donors were used to initiate and expand T-cell cultures during secondary immune response in vitro. Five of the donors yielded continuously growing T-cell cultures which showed reproducible strong lytic activities towards measles virus-infected autologous fibroblasts. Uninfected or herpes simplex virus-infected targets were weakly susceptible to these effectors. By contrast, T-cell cultures from three other seropositive donors expressed comparable lytic activities for measles virus- or herpes simplex virus-infected targets, but not for uninfected autologous targets. The five T-cell cytolytic cultures which revealed measles virus specificity also displayed human histocompatibility leukocyte antigen (HLA)-A and HLA-B restriction, i.e., were lytic for targets sharing HLA-A or HLA-B or both with them. Additionally, it was found that a monoclonal anti-HLA antibody (W6/32) could effectively block the measles virus-specific and HLA-A- and HLA-B-related lytic activities of these cytotoxic T-lymphocytes. The specificity of this blocking effect was reflected by the inefficacy of a monoclonal anti-HLA-DR antibody to block the cytotoxic T-lymphocyte-mediated lysis.

The status of human T-cell-mediated cytotoxicity specifically directed against measles virus (MV)-infected cells in vitro remains unresolved despite several studies of this phenomenon. In some earlier studies (1, 9) T-cell-enriched populations from human peripheral blood lymphocytes (PBL) failed to kill MV-infected cells, whereas antibody-dependent cell-mediated (presumably by K-cells) cytotoxicity (9, 14) or natural killer (NK) cell cytotoxicity against such targets could be shown reproducibly. Ewan and Lachmann (4) reported that T-cells from donors immune to MV can kill MV-infected targets in the absence of specific antibody; however, the effectors in this situation obviously were not analogous to the classical virus-specific cytotoxic T-lymphocytes (CTLs) because this cytotoxicity was not restricted by the products of the major histocompatibility complex, i.e., the human histocompatibility leukocyte antigens (HLAs).

Evidence that HLAs are involved in the T-cell-mediated cytotoxicity against MV-infected cells was presented in two reports. Kreth et al. (5) reported the detection of MV-specific CTLs showing HLA restriction directly in the PBL of two children with acute measles. In addition, in an in vitro study (15), the coculturing of human PBL from seropositive persons with autologous MV-infected fibroblasts resulted in the generation (besides non-T cytotoxic effectors) of MV-specific and HLA-restricted CTLs. To a large extent the reasons for the discrepant results obtained in the studies referred to above may be explained either by variations in the experimental protocol used for obtaining cytotoxic effectors or by the assaying together of heterogeneous cytotoxic effector populations with qualitative or quantitative differences in terms of lytic potential.

Earlier we reported the feasibility of obtaining exponentially growing CTL cultures from the PBL of donors sensitized to herpes simplex virus (HSV), cytomegalovirus, or Epstein-Barr virus which could mediate HLA-associated killing of the respective virus-infected targets (12). By using essentially the same approach, we have been able to generate and expand MV-specific CTLs which exerted highly efficient lytic activity against MV-infected targets that shared HLA-A or HLA-B or both with the effectors.

MATERIALS AND METHODS

Viruses. The Edmonston strain of MV (National Institutes of Health reference strain) was propagated on African green monkey kidney cells (Vero cells).

This stock was free of mycoplasma contamination and contained 10^6 PFU/ml when titrated on Vero cells.

HSV type 1 (strain Thea) was grown on monolayers of baby hamster kidney (BHK-21) cells, and the stock virus contained 3.5×10^6 PFU/0.5 ml.

Preparation of continuously growing cultures of effector T-cells. Sterile heparinized (10 IU of heparin sodium per ml) venous blood samples were obtained from eight healthy MV-seropositive volunteers. The blood samples were diluted 1:1 with phosphate-buffered saline, and mononuclear cells were harvested by Ficoll-Hypaque (Lymphoprep; Nyegaard, Oslo, Norway) density gradient centrifugation ($800 \times g$ for 30 min) by the technique of Böyum (2). The cells at the interface were removed, washed twice with phosphate-buffered saline, and suspended in growth medium (GM) containing RPMI 1640 (Flow Laboratories, Inc.), 20% heat-inactivated fetal calf serum, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 200 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. For some experiments the GM contained 10% pooled human serum. Portions (5 ml) of cell suspensions (10×10^6 mononuclear cells) from each of the eight donors were cultured on autologous fibroblast monolayers (10^6 cells) which were preinfected with MV at a multiplicity of infection of 1 for 3 days and subsequently fixed with glutaraldehyde before use. After 5 days of incubation at 37°C in a humidified atmosphere of 5% CO_2, T-cells were purified from stimulated cultures by the sheep erythrocyte rosette-depleting technique (3, 13).

Sheep erythrocytes were dissociated from the rosetted T-cell pellet by lysing for 8 to 10 min at 0°C with Tris buffer containing 0.83% ammonium chloride. T-lymphocytes obtained in this way were 95% purified as assessed by the rosette test. Portions (20 μl) of T-cell-enriched preparations (10^6 cells) were seeded in individual wells of 24-well Costar tissue culture dishes and fed with GM with pooled human serum plus 20% T-cell growth factor (TCGF; 1.5 ml per well). After 5 to 7 days, when the cells had increased in density, the bulk T-cell cultures were split to 1×10^4 cells per ml in fresh GM with TCGF. The cells used in the present cytolysis assays were maintained in culture for at least 2 months.

Preparation of TCGF. Human TCGF was prepared by stimulating human PBL with phytohemagglutinin (6). Briefly, a pooled PBL preparation from five normal individuals was cultured at a density of 5×10^6 cells per ml in GM containing 1% bovine serum albumin (GIBCO Diagnostics) instead of fetal calf serum, and 1 μg of phytohemagglutinin (PHA-P; Difco Laboratories) per ml. After 72 h at 37°C, the cell-free supernatant was removed, concentrated by 85% $(NH_4)_2SO_4$ precipitation, and then dialyzed overnight at 4°C against Tris buffer (pH 8.0). This concentrate, referred to as TCGF, was stored at −20°C until required. For some experiments, TCGF purchased from Biotest Diagnostics was used for the expansion of cultures.

Target cells. Secondary human skin fibroblast cultures were prepared in 25-cm^2 flasks by the standard procedure. Briefly, punch skin biopsies were obtained from HLA-typed individuals. The tissue was chopped with a razor blade, and the pieces (about 1 by 1 by 1 mm) were placed in 30-mm plastic petri dishes and cultured with GM plus fetal calf serum. Thrice-subcultured monolayers (approximately 5×10^5 to 10×10^5 cells) were infected with the Edmonston strain of MV at a multiplicity of infection of 1 (assayed on Vero cells). Following adsorption at 37°C for 1 h, the cells were washed and fed with fresh GM. After 5 to 6 days of incubation, when viral antigens could be detected on 85 to 90% of the infected cells by the indirect immunofluorescence test, the infected monolayers were dispersed with trypsin and labeled by suspending 10^6 cells in 0.2 ml of GM with pooled human serum containing 200 μCi (0.1 ml) of $Na_2{}^{51}CrO_4$ (specific activity, 300 to 600 mCi/mg). After incubation at 37°C for 1 h, ^{51}Cr-pulsed cells were washed three times and suspended in GM with pooled human serum (10^5 viable cells per ml). Mock-infected control fibroblasts were given medium without virus.

Cytolysis assay. Portions (100 μl; 10^4 cells) of test targets were mixed in round-bottomed plastic tubes with 100 μl of various concentrations of appropriate effector cells to give the desired effector-to-target (E:T) ratios. After centrifugation at 50 rpm for 10 min, the tubes were incubated for 4 h at 37°C and then centrifuged at 1,200 rpm for 15 min in the cold; 100 μl of supernatant was collected from each tube and counted in a gamma counter. A spontaneous ^{51}Cr release control and maximum ^{51}Cr release control were included in all of the assays and consisted of 100 μl of medium or 1% Triton X-100 (30 min), respectively, added instead of effectors. The value of total releasable ^{51}Cr from both uninfected and infected targets ranged from 80 to 90% of the incorporated isotope. The results are expressed as the percentage of specific ^{51}Cr release (mean of triplicate wells in a typical experiment) according to the formula: $100 \times (Ex - S)/(M - S)$, where Ex is the test ^{51}Cr release with effectors, S is the spontaneous ^{51}Cr release in medium, and M is the maximum releasable ^{51}Cr with detergent.

During the 4-h assay period, the leakage of ^{51}Cr from MV-infected or uninfected targets in the presence of medium ranged from 10 to 32% of the maximum releasable ^{51}Cr in different instances. The standard error of the mean seldom exceeded 4% and is omitted from the tabulated data. In some experiments data are recorded as the percent inhibition according to the formula: % inhibition of lysis = 1 − [(% lysis in presence of antibody)/(% lysis in controls)] × 100.

Cytolysis blocking experiments. For blocking experiments, W6/32 monoclonal antibody against HLA-A, HLA-B, and HLA-C (8; Sera Lab) and RITC anti-HLA-DR antibody (Becton, Dickinson & Co.) were used. Appropriately diluted antibody preparation was added to ^{51}Cr-labeled target cells (25 μl per 10^4 cells) and then incubated at 25°C for 30 min before the effector cells were added and the cytolysis assay was performed as described above.

RESULTS

Cytolytic reactivities of T-cell cultures. Continuously growing T-cell cultures originating from the PBL of eight MV-seropositive donors were tested for lytic activities in a 4-h ^{51}Cr-release assay. Table 1 shows the data from one of several experiments of this type, all of which gave similar results. T-cell cultures derived from

TABLE 1. Lytic potential of continuously growing T-cell cultures derived from the PBL of MV-seropositive donors

Donor[a]	% Specific ^{51}Cr release (4-h assay) from autologous fibroblasts infected with:[b]					
	Uninfected		HSV type 1		MV	
	40:1	10:1	40:1	10:1	40:1	10:1
1	1.7	−5.4	5.7	1.1	66.9	40.4
2	1.1	0.2	4.4	−0.4	43.5	18.9
3	0	0.9	10.9	−1.9	46.3	21.3
4	0	−3.0	4.6	−2.3	64.4	44.7
5	1.8	−2.9	9.8	2.1	44.3	20.1
6	1.3	0.8	36.7	16.4	44.2	19.6
7	2.9	−0.4	31.8	19.7	46.7	27.6
8	4.1	−2.1	44.1	26.6	54.9	23.8

[a] The cultures were kept in GM with TCGF for 45 to 59 days until the time of assay. The extent of lysis mediated by these effectors against K562 targets included as controls in each assay was uniformly below 5% in all instances.

[b] Secondary human skin fibroblasts were used. Cultures from HLA-typed individuals were inoculated with MV or HSV type 1 at a multiplicity of infection of 1. After virus adsorption at 37°C for 1 h, the monolayers were washed and incubated for 5 to 6 days (MV) or 6 h (HSV) before use as targets. Assays were done at E:T ratios of 40:1 and 10:1.

five individuals (donors 1, 2, 3, 4, and 5) showed a strong lytic effect for MV-infected but not for HSV-infected autologous fibroblasts. By contrast, cultured effector T-cells from each of the remaining three donors (donors 6, 7, and 8) induced comparable lysis of MV- or HSV-infected autologous targets. They also induced significant lysis of MV- or HSV-infected non-HLA-identical targets (data not shown). None of these eight T-cell cultures lysed uninfected autologous targets to a significant extent. Apparently, the data reflect marked variations in the lytic levels among the five CTL cultures which displayed specificity for MV. For instance, CTL from donors 1 and 4 induced 40.4 and 44.7% of specific ^{51}Cr release, respectively, from test targets at an E:T ratio of 10:1. In contrast, for this level of lysis, each of the other CTL cultures (i.e., from donors 2, 3, and 5) required an E:T ratio of 40:1.

HLA restriction in CTL cultures displaying MV specificity. It is now appreciated that virus-specific CTLs, at least in the influenza virus (7) and herpesvirus (11, 12) systems, may demonstrate the HLA restriction phenomenon. To determine whether the MV-specific lytic effect shown by five of the CTL cultures was also HLA related, the cultures were tested as effectors against a selected panel of MV-infected HLA-matched or HLA-mismatched targets. The results of a representative experiment (Table 2) show that each of the five CTL cultures was strongly lytic for MV-infected autologous, HLA-A-matched, or HLA-B-matched targets. With each of the five effector cultures, the specific lysis of MV-infected autologous targets was always of higher magnitude than the lysis of allogeneic HLA-A- or HLA-B-matched targets. These CTLs proved ineffective in exerting a specific lytic effect against MV-infected HLA-A and HLA-B non-identical targets or against targets that shared HLA-C or HLA-DR or both with the effectors. The lack of sensitivity to lytic attack of these CTLs cannot be attributed to an inherent insusceptibility to cell-mediated lysis per se, because in separate experiments (data not shown) they were adequately lysed by allogeneic CTLs.

Blocking of anti-MV and HLA-related lytic activities of CTL cultures by W6/32 monoclonal anti-HLA antibody. Earlier, it was shown that monoclonal anti-HLA antibodies may inhibit the lysis mediated by influenza virus-specific and HLA-restricted CTLs (7) or alloreactive CTLs (10). Attempts were made to determine whether the lytic attack by the MV-specific CTL cultures on relevant targets could be blocked by two of the available monoclonal anti-HLA antibodies, W6/32 and RITC. Monoclonal W6/32 antibody reacts with framework determinants of HLA-A, HLA-B, and HLA-C, and monoclonal RITC recognizes common determinants on HLA-DR.

TABLE 2. MV-specific and HLA-restricted lytic activities of T-cell cultures derived from MV-seropositive donors

Shared HLA of target panel with effectors[a]	% Specific lysis (4-h assay; E:T ratio, 35:1) of MV-infected targets by CTL cultures derived from donor:[b,c]				
	1	2	3	4	5
All (autologous)	49.4	35.5	39.6	40.9	36.3
HLA-A, HLA-B	32.9	30.6	31.9	37.6	30.8
HLA-C, HLA-DR	9.2	4.9	3.6	7.9	4.9
HLA-A2	33.8	—[d]	30.4	33.9	—
HLA-B7	27.8	29.4	—	32.1	—
HLA-Bw35	—	30.7	—	—	—
HLA-B27	—	—	29.6	—	—
HLA-B8	—	—	—	—	27.6
None	1.8	3.6	5.9	8.1	3.5

[a] The target panel was a random selection. The effectors displayed negligible specific lytic activity for uninfected HLA-matched or HLA-mismatched targets.

[b] The mean spontaneous release from different MV-infected targets ranged from 9 to 20% in 10 separate experiments.

[c] HLA serotypes of the CTL donors were as follows. Donor 1: A2, A19; B7, B15; Cw3; DR2, DR6. Donor 2: A3, A11; B7, Bw35; Cw1; DR2, DR6. Donor 3: A2, A11; B27, Bw35; Cw1; DR1, DR4. Donor 4: A2, A19; B7, B40; Cw3; DR2, DR$^-$. Donor 5: A1, A9; B8, B12; Cw4; DR3, DR$^-$.

[d] —, Not done.

W6/32 antibody was able to block the lytic effect at two different dilutions (Table 3). In contrast, under identical conditions, RITC antibody failed to affect the lytic level to any appreciable extent even when used at a relatively low dilution. These results were reproducible in several separate experiments performed at different time intervals.

DISCUSSION

In the present work, long-term T-cell cultures were initiated from the PBL of eight MV-seropositive donors and assayed for their lytic potential in a short-term ^{51}Cr-release assay. Five of the donors yielded cultures which displayed significant selective lytic activity towards MV-infected, but not HSV-infected or uninfected, autologous fibroblasts. Surprisingly, cultures originating from a second set of three donors showed comparable lytic activities towards MV- and HSV-infected autologous targets, whereas uninfected targets were insensitive. As the cytolytic potential of the cultures derived from these three donors was neither virus specific nor HLA restricted, classical T-cells were most likely not effective in these instances. If it is true that the so-called NK-cells with "anomalous" cytotoxic capacity reside in the T-cell-enriched fraction, then the lysis mediated by the cultures derived from the second set of donors may be assigned to NK-cell activity. However, this argument is weakened by the fact that these cultures, unlike conventional NK-cells, lacked lytic activity for normally NK-sensitive cell lines such as K562. It may be argued that infection with MV or HSV type 1 leads to the expression of host-specified surface structures (antigen?) which serve as targets for these effectors. Alternatively, these effector cultures may be composed of mixtures of clones with heterologous specificities, some against MV and some directed against a structure(s) which may bear coincidental identity with any of the several HSV-encoded determinants. Additional studies with cloned effectors and other experimental strategies are required before these possibilities may be excluded. These studies are presently underway.

The HLA-related lytic effect of five individual T-cell cultures which initially showed MV specificity was established by testing them against a selected panel of HLA-matched or HLA-mismatched virus-infected targets in a 4-h ^{51}Cr-release assay. Thus, each of the five CTL cultures was strongly lytic for MV-infected autologous or HLA-A- or HLA-B-identical targets, but not for mismatched test targets. No significant lytic effect was noticed on uninfected targets whether or not they shared HLA-A or HLA-B specificities with the effectors. Sharing of HLA-C or HLA-DR specificities alone did not induce adequate lysis.

Finally, a monoclonal antibody against HLA-A, HLA-B, and HLA-C, but not against HLA-DR, was able to block efficiently the specific lytic effect in the present system. In fact, McMichael et al. (7) showed that the lytic effect of influenza virus-specific CTLs can be blocked by monoclonal anti-HLA antibodies. The blocking effect observed with a monoclonal anti-HLA antibody indicates that virus-specific CTLs may recognize the target HLA molecules per se. Alternate interpretations for this blocking phenomenon could be steric hindrance by bound antibody or that the effectors recognize the determinant(s) of some unknown cell surface molecule(s) displaying coincidental cross-reactivity with the test antibody which was found to be inhibitory. Although two earlier studies (5, 15) demonstrated the ability of MV to elicit MV-specific and HLA-restricted CTLs, the present study cannot technically be compared with them. In the study by Kreth et al. (5), MV-specific and HLA-restricted CTLs were detected directly in the PBL of two children 4 to 10 days after the appearance of rash. Wright and Levy (15) found that coculturing the PBL from seropositive donors with autologous MV-infected fibroblasts results in the generation (besides non-T cytotoxic effectors) of MV-specific and HLA-restricted CTLs.

To generate CTLs with dual specificity in the current study, we first exposed PBL from MV-seropositive donors to specific antigen for 5 to 7

TABLE 3. Capacity of monoclonal antibody against HLA-A, HLA-B, and HLA-C (W6/32) to inhibit the lytic attack of MV-specific and HLA-restricted CTL cultures

Antibody	Antibody dilution	% Specific ^{51}Cr release (4-h assay; E:T ratio, 35:1) from MV-infected autologous targets by CTL cultures from donor:[a]			
		3[b]		4[b]	
		Expt 1	Expt 2	Expt 1	Expt 2
None		38.3	32.7	46.2	40.8
W6/32	1:10	4.2	7.8	3.9	5.6
	1:50	17.6	19.2	12.8	15.7
RITC	1:4	35.0	30.7	47.4	36.4
	1:10	36.4	33.1	42.8	38.4
NMA[c]	1:4	32.4	35.1	40.7	40.1

[a] A total of 10^4 ^{51}Cr-labeled fibroblasts, infected for 5 days with MV at a multiplicity of infection of 1, were incubated with 25 μl of W6/32 or RITC antibody at 25°C for 30 min, and then the effectors were added. Antibody was present during the assay. Antibody lots were obtained as ascites fluid.

[b] HLA serotypes for donors 3 and 4 are given in Table 2, footnote *c*.

[c] NMA, Normal mouse ascites fluid.

days and then used highly enriched T-cell populations to propagate them in the presence of TCGF. This protocol for generating CTLs was adapted from an earlier study designed to obtain herpesvirus-specific CTLs (12). The novelty of this method is the ability to expand and maintain human CTL populations which express virus specificity and HLA restriction in culture. Of particular note was the observation that bulk T-cell cultures derived in this manner from the PBL of three of the eight MV-seropositive donors, although strongly lytic for various targets, showed no evidence of MV specificity. We are presently investigating why MV-specific and HLA-restricted secondary CTLs can be reproducibly generated from the PBL of some seropositive donors but not from others. In conclusion, the present study has shown the feasibility of generating and selectively expanding MV-specific and HLA-restricted CTLs in vitro. Such cultures should be useful for investigating the nature of the target antigen(s) involved in the CTL recognition phenomenon. The question of whether specific CTLs are functionally active in vivo under certain clinical conditions remains to be elucidated.

LITERATURE CITED

1. **Ault, K. A., and H. L. Wiener.** 1979. Natural killing of measles-infected cells by human lymphocytes. J. Immunol. **122**:2611–2616.
2. **Böyum, A. L.** 1968. Separation of leukocytes from the blood and bone marrow. Scand. J. Clin. Lab. Invest. **21**(Suppl.97):107–109.
3. **Chess, L., R. P. MacDermott, and S. F. Schlossmann.** 1974. Immunologic functions of isolated human lymphocyte subpopulations. I. Quantitative isolation of human T and B-cells and response to mitogens. J. Immunol. **113**:1113–1121.
4. **Ewan, P. W., and P. J. Lachmann.** 1977. Demonstration of T-cell and K-cell cytotoxicity against measles-infected cells in normal subjects, multiple sclerosis and subacute sclerosing panencephalitis. Clin. Exp. Immunol. **30**:22–31.
5. **Kreth, H. W., V. Ter Meulen, and G. Eckert.** 1979. Demonstration of HLA restricted killer cells in patients with acute measles. Med. Microbiol. Immunol. **165**:203–214.
6. **Kurnick, J. T., K. Grönick, A. K. Kimura, J. B. Lindblom, V. T. Skoog, O. Sjöberg, and H. Wigzell.** 1979. Long-term growth in vitro of human T-cell blasts with maintenance of specificity and function. J. Immunol. **122**:1255–1260.
7. **McMichael, A. J., P. Parham, F. M. Brodsky, and J. R. Pilch.** 1980. Influenza virus specific cytotoxic T lymphocytes recognize HLA molecules. Blocking by monoclonal anti-HLA antibody. J. Exp. Med. **132**:195–203.
8. **Parham, P., C. J. Barnstable, and W. F. Bodmer.** 1979. Use of a monoclonal antibody ($W6/_{32}$) in structural studies of HLA-A, B, C, antigens. J. Immunol. **123**:342–349.
9. **Perrin, L. H., A. Tishon, and M. B. A. Oldstone.** 1977. Immunologic injury in measles virus infection. III. Presence and characterization of human lymphocytes. J. Immunol. **118**:282–290.
10. **Reiss, C. S., M. E. Hemler, V. H. Englehardt, J. W. Mier, J. L. Strominger, and S. J. Burakoff.** 1980. Development and characterization of allospecific long-term human cytolytic T-cell lines. Proc. Natl. Acad. Sci. U.S.A. **77**:5432–5436.
11. **Rickinson, A. B., L. E. Wallace, and M. A. Epstein.** 1980. HLA-restricted T-cell recognition of T-lymphocyte cytotoxicity to Epstein-Barr virus. Nature (London) **283**:865–867.
12. **Sethi, K. K., I. Stroehmann, and H. Brandis.** 1980. Human T-cell cultures from virus-sensitized donors can mediate virus-specific and HLA-restricted cell lysis. Nature (London) **286**:718–720.
13. **Werner, W., C. Bianco, and V. Nussenzweig.** 1973. Enhanced binding of neuraminidase-treated sheep erythrocytes to human T lymphocytes. Blood **42**:939.
14. **Whittle, H. C., and J. Werblinska.** 1980. Cellular cytotoxicity to measles virus during natural measles infection. Clin. Exp. Immunol. **42**:136–143.
15. **Wright, L. L., and N. L. Levy.** 1979. Generation on infected fibroblasts of human T and non-T lymphocytes with specific cytotoxicity, influenced by histocompatibility against measles virus-infected cells. J. Immunol. **122**:2379–2387.