α-chain sequence are not more homologous to Ig_H chains than they are to Ig_L chains. Furthermore, our data suggest that α-chain genes are located on chromosome 14 proximal to 14q22. Since Ig_H chain genes have been mapped to 14q32(5), this would place these two loci ≥45 centimorgans (cM) apart. It would be difficult to detect such a relatively weak linkage by classical genetic analysis in that genes 50 cM apart behave as independently assorting elements. Whereas the Ig_H chain and T-cell receptor α-chain loci may be the products of an ancient gene duplication, the actual evolutionary relation between these two loci still needs to be determined. Perhaps examination of these genes in several other species will clarify this point.

The second point of interest regarding chromosome 14 concerns tumors of T-cell origin. Cytogenetic studies have been performed on cells from patients with T-cell malignancies, such as chronic lymphocytic leukemia. T-cell lymphoma, and lymphosarcoma (*11*). Although several chromosomal abnormalities can be seen in the malignant cells derived from these patients, a common feature in many of these T-cell tumors is a break involving chromosome 14. The breakpoint has been located in bands 14q11 → 13 and is associated with chromosome inversions and translocations (*12*). The involvement of bands 14q11 → 13 in these tumors, had led Hecht *et al.* (*12*) to propose that genes relating to T-cell function are encoded at this site. Our data are consistent with the possibility that the human α-chain locus is located in bands 14q11 → 13. Given the precedent of chromosome abnormalities involving Ig genes in B-cell tumors (*13*), there remains the possibility that the chromosome 14 rearrangements seen in some T-cell tumors involve α-chain genes.

CAROL JONES
HELVISE G. MORSE
FA-TEN KAO
Eleanor Roosevelt Institute for Cancer Research and *Department of Biochemistry, Biophysics and Genetics, University of Colorado, Health Sciences Center, Denver 80262*

AMY CARBONE
EDWARD PALMER*
Department of Medicine, National Jewish Hospital and Research Center, 3800 East Colfax Avenue, Denver, Colorado 80206 and *Department of Microbiology and Immunology, University of Colorado Health Sciences Center*

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* To whom correspondence should be addressed.

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Human T-Cell Clones from Autoimmune Thyroid Glands: Specific Recognition of Autologous Thyroid Cells

Abstract. *The thyroid glands of patients with autoimmune diseases such as Graves' disease and certain forms of goiter contain infiltrating activated T lymphocytes and, unlike cells of normal glands, the epithelial follicular cells strongly express histocompatability antigens of the HLA-DR type. In a study of such autoimmune disorders, the infiltrating T cells from the thyroid glands of two patients with Graves' disease were cloned in mitogen-free interleukin-2 (T-cell growth factor). The clones were expanded and their specificity was tested. Three types of clones were found. One group, of T4 phenotype, specifically recognized autologous thyroid cells. Another, also of T4 phenotype, recognized autologous thyroid or blood cells and thus responded positively in the autologous mixed lymphocyte reaction. Other clones derived from cells that were activated in vivo were of no known specificity. These clones provide a model of a human autoimmune disease and their analysis should clarify mechanisms of pathogenesis and provide clues to abrogating these undesirable immune responses.*

Autoimmune diseases involving the thyroid gland are characterized by a variety of humoral and cell-mediated immunological phenomena (*1*). These lead to either tissue destruction, with consequent inhibition of function as in Hashimoto's thyroiditis (*2*) or primary myxedema (*3*), or to hyperstimulation of hormone secretion and hypertrophy of the gland as in Graves' disease (*4*) and in

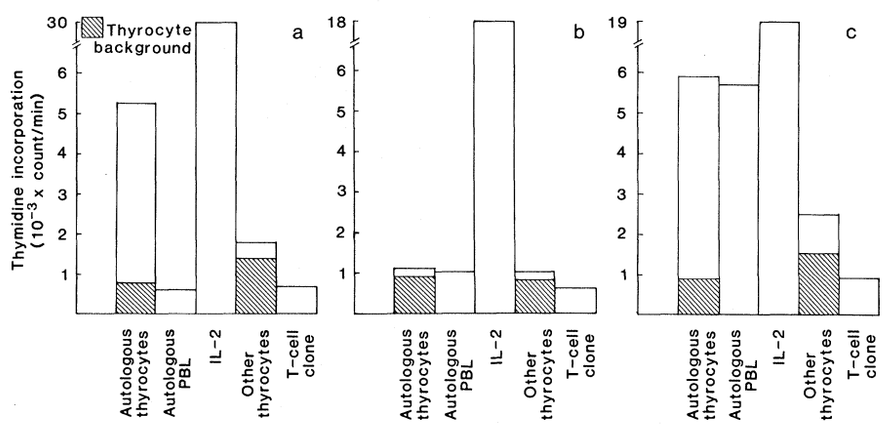

Fig. 1. Proliferative response to one clone from each of the groups. (a) Clone 17 is thyrocyte-specific. Response of T cells alone and to autologous thyrocytes is clearly seen with the thyrocyte background (no T cells, shaded); 10^4 T cells and 10^4 thyrocytes or peripheral blood cells per well, response assayed as in Table 1. (b) Clone 15 does not respond to thyroid or blood cells, only to IL-2. (c) Clone 51 is an autologous MLR clone, responding equally to autologous thyroid (autol thyr) and peripheral blood mononuclear cells (pbmnc).

certain cases of unexplained goiter (*5*). The histology of autoimmune thyroid glands is characterized by B- and T-lymphocyte infiltration, and it was recently shown that the thyroid follicular cells, in contrast to those of normal glands, strongly express MHC class II (HLA-DR) molecules (*6*). We have proposed that this aberrant expression of HLA-DR may be involved in the initiation and maintenance of autoimmune disease (*7, 8*). Since these diseases usually involve immunoglobulin G antibodies, which are T helper cell dependent (*9*), it is likely that T helper cell activity is of paramount importance in their pathogenesis. Because the infiltrating T lymphocytes and the epithelial cells are accessible at operation, we decided to study the thyroid glands from two patients (A and B) with Graves' disease undergoing partial thyroidectomy.

Activated T cells express several new receptors, including that for interleukin-2 (IL-2, or T-cell growth factor) (*10*) that can be detected by the anti-Tac monoclonal antibody (*11*). Thus, in order to clone intrathyroid T cells that were activated in vivo, we cultured isolated intrathyroid mononuclear cells (6 to 10 percent Tac^+) in mitogen-free IL-2 for 7 days, in the absence of any antigen, before cloning them in Terasaki plates (0.3 cells per well) and allowing subsequent expansion in IL-2. After 4 weeks of culture, the specificity of the T-cell clones was tested by a proliferation assay in which we used autologous thyroid follicular epithelial cells (thyrocytes) that had been cryopreserved (Table 1).

Three types of T-cell clones were isolated (Fig. 1). Clone 17 was typical of thyrocyte-specific cells, recognizing only autologous thyroid. The response of this clone to autologous blood cells, or to histoincompatible thyrocytes from other patients with Graves' disease spontaneously expressing HLA-DR antigens (*6*), was negligible, the isotope incorporation being no greater than that of T cells cultured alone. Clone 15 was typical of

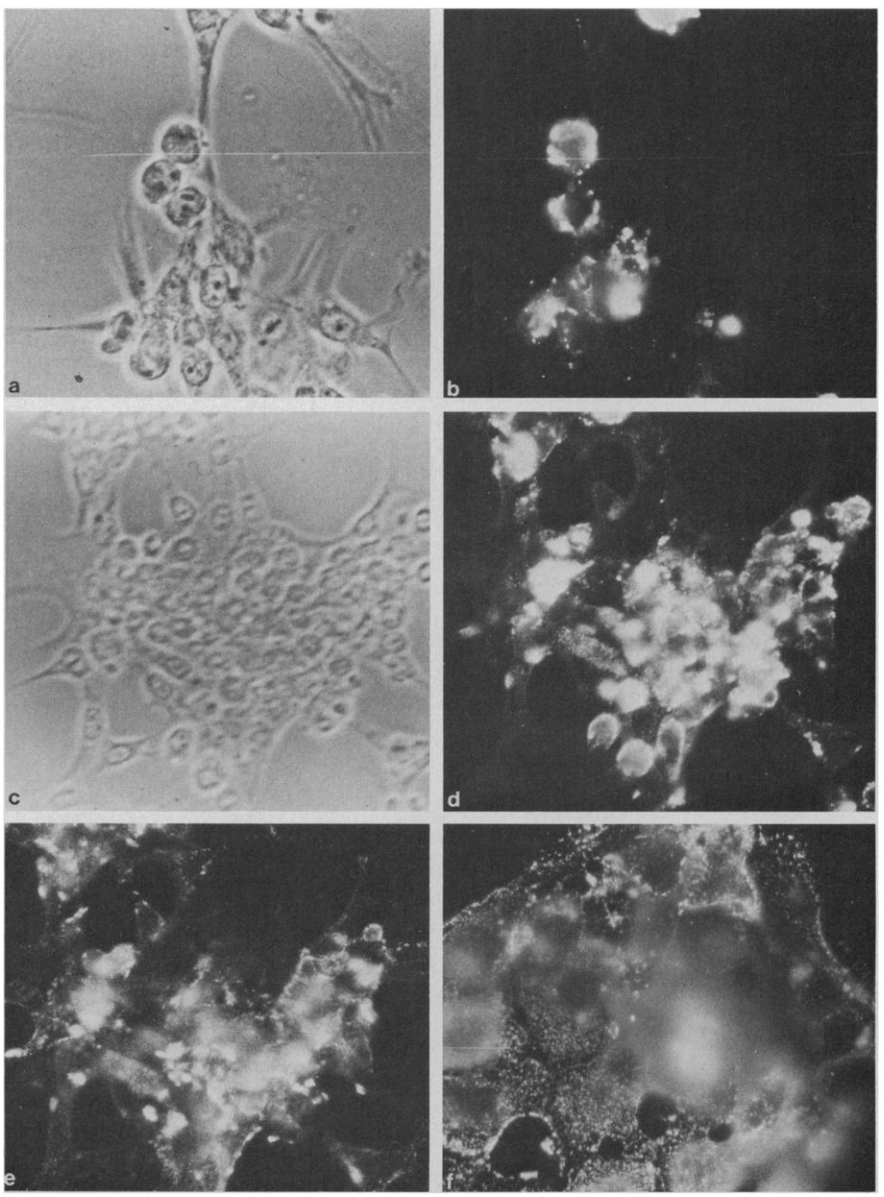

Fig. 2. Phase contrast and indirect fluorescence photomicrographs of the coculture of thyrocytes with autoreactive clones. (a) Phase contrast of clone 17 from patient A, showing the adherence of the autoreactive T-cell clone to the thyroid epithelial cells. Cells were plated onto a cover slip in a Linbro 24-well plate at a concentration of 10^5 per milliliter. After 2 days the cover slip was washed to remove unattached cells. No IL-2 was added in the culture media. Recombinant IL-2 does not induce DR expression (*26*). (b) Photomicrograph of same cells as in (a). Viable cells were incubated with anti-Tac and stained with fluorescein-labeled rabbit antiserum to mouse immunoglobulin G. The cells on the cover slip were then fixed in acetone, mounted, and viewed under a Zeiss type III microscope equipped with epi-illumination. Only the T cells are brightly stained (thyrocytes are negative), thus showing the surface expression of IL-2 receptors. The IL-2 receptor expression and enlargement (blast transformation?) of the T cell of clone 17 indicates that it is restimulated by contact with autologous thyrocytes, in agreement with the functional assay (Fig. 1a). (c) Phase-contrast photomicrograph of clone 17 from patient A, showing adherent T cells after 2 days of culture on a cover slip. (d) Same preparation as in (c), incubated with anti-DR and anti-DP (DA 6.231) (*27*) and treated as in (b). Both T cells and autologous thyrocytes are positive. The granular membrane fluorescence of the latter is similar to that observed previously (*28*). Thyrocytes from this patient spontaneously expressed DR when first isolated, and in this preparation appear more positive after they have adhered to T cells. (e) Same preparation as in (c) and (d), stained by double immunofluorescence technique with a serum from a patient with Hashimoto's thyroiditis (diluted 1:10) known to contain autoantibodies to microvillar-microsomal thyroid antigens as described (*29*). The reaction was revealed with a conjugate of rhodamine with rabbit antiserum to human immunoglobulin G. The T cells are negative and only thyrocytes are stained, indicating no cross-reactivity among reagents obtained from different species. Visual comparison between this preparation and that in (d) indicates that microvillar surface antigens are distinct entities from DR molecules expressed on these cells (*28*). There is a minor degree of overlap due to nonspecific staining or autofluorescence lypofuscin granules (larger spots). (f) Photomicrograph of a parallel culture where thyrocytes from patient A were plated on a cover slip at same time as in (c), but not cocultured with T cells from clone 17. The preparation was stained as in (e). Note the greater intensity of staining than in (e), indicating a decreased expression of microvillar antigens on thyrocytes cocultured with autoreactive T-cell clones. This phenomenon, although difficult to quantify, has been noted repeatedly with clone 17 and other clones such as 1 and 13.

cells of unknown specificity, not recognizing autologous thyrocytes or blood cells. Clone 51 reacted like an "autologous mixed lymphocyte response cell" (*12, 13*), recognizing both autologous HLA-DR$^+$ thyrocytes and blood cells. That these cells are of major importance in producing lymphokines in autoimmune disease was proposed recently (*12, 13*) and is confirmed by the fact that such cells expressing IL-2 receptors were present in the target tissue and were activated in vivo.

Many specific clones were identified from both patients by means of a 3-day assay in which we used a 1:1 ratio of cloned cells to thyrocytes [as is routinely used for isolating clone specific for influenza virus (*14*)]. About 9 of 60 clones derived from patient A and 10 of 100 clones derived from patient B responded specifically to autologous thyroid but not to mismatched DR$^+$ thyroid cells or autologous peripheral blood lymphocytes (PBL) (containing class II$^+$ cells). However, this may be an underestimate because we had a relatively small number of autologous thyrocytes from the patients and could not perform rigorous time course and antigen dose-response studies. Thus some of the T-cell clones negative here may have responded under different stimulating conditions. Clones growing well in culture were selected for further analysis, and some were recloned.

The proliferation of T cells from mice (*15*) or humans (*16*) in response to antigen is readily blocked by antibodies to MHC class II antigens. To verify that the proliferative response detected to autologous thyrocytes was a typical antigen-specific proliferative response and not a hormonal or mitogenic effect of, say, released thyroid hormones, we evaluated the effect of monoclonal antibodies to MHC class II antigens (anti-HLA-DR, anti-DC, and anti-SB, now termed, re-

Table 1. Summary of the autoreactive T-cell clones. Thyroid tissue, aseptically removed at surgery, was divided into two parts of equal dimension. One part was digested with collagenase type IV (Worthington) (5 mg/ml) for 3 hours at 37°C in the absence of serum. The digest was filtered through a 200-μm mesh. The thyroid epithelial cells (thyrocytes) were counted, frozen, and stored in liquid nitrogen until used (*21*). The other part was teased apart in cold phosphate-buffered saline and filtered through a 200-μm mesh to release mononuclear cells; the suspension was purified on a Lymphopaque gradient. The lymphocytes thus obtained were plated at 0.5×10^6 per milliliter in RPMI 1640 medium supplemented with 10 percent type A$^+$ pooled human serum and 25 percent MLA-144 supernatant (*30*) as a mitogen-free source of IL-2, and cultured for 7 days. They were then enriched on a Ficoll-Hypaque density gradient and plated at 0.3 cells per well in sterile 60-well Microtest II trays (Falcon) in the presence of IL-2, with autologous irradiated PBL (5×10^3) being used as a source of filler cells (*14*). Interleukin-2 was prepared as described (*14*). The wells that showed positive growth at day 7 were transferred to 96-well round bottom microtiter trays (Falcon) and cultured with autologous irradiated PBL (5×10^4 per well) in the presence of IL-2. After a further 7 days in culture, the clones were transferred to 24-well trays containing IL-2 and autologous irradiated thyrocytes (5×10^5 per well). In the preliminary screen clones were examined for antigen specificity in a 72-hour assay by using [^{3}H]thymidine (New England Nuclear). Individual clones, diluted 1:200, from 24-well trays ($\sim 5 \times 10^3$ cells per well) were cultured with autologous irradiated thyrocytes (about 1×10^4 per well) in a total volume of 200 μl. Because of the adherence of thyrocytes, the exact number plated varied. The positive clones were studied, with autologous thyrocytes or PBL, HLA mismatched thyrocytes (all of them at 1×10^4 per well), and IL-2 (25 percent supernatant of phytohemagglutinin-stimulated PBL). Clone 17 was assayed repetitively to verify the specificity of the assay and of the cells. All the experiments were performed in triplicate in a 72-hour proliferative assay. Cultures were exposed to 1 μCi of [^{3}H]thymidine during the last 10 hours. Incorporation was measured by liquid scintillation spectroscopy. Results are expressed as mean counts per minute ± standard error (SE). When tested, the phenotype was always T4$^+$ (with Leu 3, Becton Dickinson); ND, not done. All were T8 negative and T3 positive. The background counts of thyrocytes cultured alone (which do take up some thymidine) are indicated at the bottom of the table. Three experiments (superscripts a, b, and c) with patient A (HLA-DR 2,3) are shown, and the values for isotope incorporation by autologous thyrocytes (TEC) and unrelated TEC were: (a) autologous 821 ± 407, unrelated TEC 1869 ± 804; (b) autologous 606 ± 234, unrelated TEC 228 ± 92; (c) autologous 912 ± 438, unrelated TEC not determined. On patient B (HLA-DR 4,5), autologous TEC 550 ± 137, unrelated TEC 536 ± 75. In the last experiment, four DR unrelated thyrocyte preparations (DR 1-10, DR 4-5, DR 4/X, DR 3/X) were used, which did not stimulate clone 17 of patient A. Backgrounds (thyrocytes alone) are indicated in brackets.

Clone	Reactivity	Phenotype	Response to stimulus (count/min ± SE)				IL-2
			Autologous		Unrelated		
			TEC	PBL	TEC		
Patient A							
1	+	T4	2238 ± 510	680 ± 117	602 ± 28		22678 ± 2049[b]
3	?	ND	1559 ± 364	631 ± 252	931 ± 595		19913 ± 4676[b]
12	?	T4	1634 ± 51	1756 ± 454	1308 ± 373		43384 ± 3152[a]
13	+	T4	4675 ± 495	1494 ± 162	1810 ± 166		12090 ± 1086[a]
15	?	T4	1160 ± 184	1110 ± 270	990 ± 289		18598 ± 1392[b]
17	+	T4	5144 ± 389	605 ± 68	1838 ± 273		30640 ± 1934[a]
19	AMLR	ND	5581 ± 1773	5980 ± 565	1677 ± 401		2470 ± 1922[a]
51	AMLR	T4	5791 ± 1367	5541 ± 1861	2688 ± 1794		18973 ± 4488[a]
Patient B							
8	+	ND	4033 ± 203	288 ± 61	306 ± 97		8285 ± 1009
9	+	T4	4416 ± 454	790 ± 155	530 ± 207		1503 ± 106
20	?	ND	1108 ± 381	399 ± 138	1337 ± 217		14992 ± 382
21	?	ND	1202 ± 117	764 ± 112	302 ± 65		16398 ± 1076
49	+	T4	2980 ± 93	930 ± 303	301 ± 65		2057 ± 124
61	+	ND	2932 ± 231	378 ± 24	432 ± 37		1663 ± 72
69	AMLR	T4	2372 ± 8	1946 ± 433	513 ± 93		1058 ± 76
89	+	ND	3104 ± 233	434 ± 32	225 ± 35		2629 ± 174
Patient A							
17	+	T4	2374 ± 173	402 ± 109	ND		4359 ± 288[c]
			5638 ± 2107	605 ± 127	2056 ± 1253		1801 ± 345[b]
			5144 ± 389	605 ± 68	1838 ± 273		30640 ± 1934[a]
				Background		Background	
17	+	T4	3718 ± 912	(1050 ± 113)	686 ± 115	(597 ± 129)	3051 ± 30
					1324 ± 468	(1199 ± 188)	
					2052 ± 307	(1464 ± 102)	
					2358 ± 454	(2707 ± 350)	

Table 2. Inhibition of autoreactive response by monoclonal antibodies to MHC class II antigens. Clone 17 (10^4 cells per well) was stimulated with $\sim 10^4$ autologous (DR 2,3) thyrocytes (TEC) or allogeneic (DR 1,10) thyrocytes in 96-well flat bottom culture plates for 3 days, in triplicate. Cultures were exposed to 1 μCi of [^{3}H]thymidine, and incorporation was measured by liquid scintillation spectroscopy. Results are expressed as arithmetic mean counts per minute ± SE; ND, not done. Antibodies used (10 to 20 μg/ml) were: (a) a mixture of anti-DR, anti-DQ, anti-DP [DA2 (*31*), HIG78 (*27*), DA 6.231 (*27*)]; (b) anti-DR and anti-DP (DA 6.231); (c) anti-DR (19.26.1) (*32*); (d) anti-T cell p67 (G19-3.2 T1 antigen); and (e) anti-B-cell p32 (TH7, B1 antigen). Numbers in parentheses indicate the incorporation by thyrocytes alone (background).

Response of clone 17 with	Proliferation (count/min ± SE)	
	Experiment 1	Experiment 2
		845 ± 106
TEC	5144 ± 355 (821 ± 705)	3718 ± 912 (1050 ± 113)
Allogeneic TEC	1839 ± 273 (1869 ± 1328)	686 ± 115 (597 ± 129)
TEC plus anti-II	815 ± 107[a]	772 ± 55[b]
TEC plus anti-II	ND	1080 ± 77[c]
TEC plus anti-T p67[d]	ND	3503 ± 885
TEC plus anti-Bp32[e]	ND	3855 ± 91
PBL	605 ± 127	ND
IL-2	30640 ± 1934	3051 ± 30

spectively, anti-DQ and anti-DP). Table 2 shows that the proliferative response induced by thyrocytes in vitro was abrogated by the antibodies to class II antigens as were responses induced by other antigen-presenting cells (*16*).

To determine the type of T cell that had been cloned from patients A and B, we performed a phenotypic analysis using monoclonal antibodies. All the cloned cells appeared to be of the helper phenotype since they stained with Leu-3 (T4 antigen) and UCHT1 (*17*) (T3 antigen) but not with UCHT4 (*18*) (T8 antigen). However, some T cells with the T4 phenotype are MHC class II restricted killer cells (*19*) and others release interleukins but do not help (*20*). Because the cloned T cells adhered to thyrocytes in coculture but did have cytotoxic effects (Fig. 2) they are not killer cells. However, during the coculture experiments the thyrocytes to which the T cells adhered remained viable and their MHC class II expression was augmented (Fig. 2d) compared with the DR expression noted when the cells were originally isolated; furthermore, their expression of microvillar-microsomal antigens [characteristic thyroid surface autoantigens (*21*)] was much reduced (compare Fig. 2, e and f). The antigens recognized by these cloned T cells is not yet known. The present results suggest that these T-cell clones can mediate directly some of the known pathology of autoimmune thyroid disease, such as aberrant MHC class II expression, it is conceivable that these cells include the ones that help the autoreactive B cells to produce the observed autoantibodies. To our knowledge this is the first report of T lymphocytes cloned from a human organ affected by autoimmune phenomena selectively recognizing the appropriate target tissue.

Because the cloned T cells express IL-2 receptors (Tac antigen) and selectively recognize, adhere to, and grow on thyrocytes specifically, these results suggest that thyrocytes in Graves' disease may act as antigen-presenting cells that present their own surface autoantigens. With the use of viral antigens we have demonstrated that MHC class II^+ thyrocytes can present synthetic peptide antigens from the hemagglutin molecule to influenza-specific T-cell clones but do not react to the whole virus (*22*).

Recently, uncloned lines of T cells specific for the acetylcholine receptor were induced in vitro by antigen from peripheral blood cells of patients with myasthenia gravis (*23*). This differs from the work reported here, however, in that the T cells we used were obtained from the affected organ (not blood), and the cells cloned were only those that had been activated in vivo. Both studies confirm the importance of abnormalities of the T-cell network in the pathogenesis of antibody-mediated autoimmunity, and of the importance of the cloning approach to establish the pathogenesis of these diseases.

This demonstration of human autoreactive T-cell clones provides an in vitro model of the initiation of a human autoimmune disease. Closer analysis of these clones will permit us to define in detail the mechanisms underlying the pathogenesis of this disease, for example, the nature of the mediators involved in triggering B cells, the moieties inducing MHC class II expression, and the MHC class II specificities recognized. In the same way as it has been suggested that B-cell receptors and the idiotype network (*24*) may be involved in autoimmunity, we have now the means of determining directly whether T-cell receptors and a receptor network are of importance in the generation of organ-specific autoimmune diseases. As shown in animal models (*25*), the use of such clones should provide clues to the possible means of abrogating undesirable autoimmune responses, either specifically or nonspecifically.

MARCO LONDEI
Imperial Cancer Research Fund, Tumor Immunology Unit, Department of Zoology, University College, London WC1E 6BT
G. FRANCO BOTTAZZO
Department of Immunology, Middlesex Hospital Medical School, London W1
MARC FELDMANN
Imperial Cancer Research Fund, Tumor Immunology Unit, Department of Zoology, University College

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Genotoxicity of Formaldehyde in Cultured Human Bronchial Fibroblasts

Abstract. *Formaldehyde, a common environmental pollutant, inhibits repair of* O^6*-methylguanine and potentiates the mutagenicity of an alkylating agent,* N*-methyl-*N*-nitrosourea, in normal human fibroblasts. Because formaldehyde alone also causes mutations in human cells, the compound may cause genotoxicity by a dual mechanism of directly damaging DNA and inhibiting repair of mutagenic and carcinogenic DNA lesions caused by other chemical and physical carcinogens.*

Formaldehyde is a ubiquitous environmental pollutant found in many occupational settings and in tobacco smoke, consumer products, and exhaust from gasoline and diesel combustion (*1*). The compound can also be generated by oxidative metabolism of many xenobiotics (*1*). For example, the carcinogen *N*-nitrosodimethylamine (DMNA) is metabolized to equimolar concentrations of methyldiazonium ion and formaldehyde. Formaldehyde causes single-strand breaks in DNA and DNA-protein cross-links; inhibits DNA excision repair in human cells (*2, 3*); is mutagenic in *Drosophila* larvae, bacteria, and fungi (*4*); is a respiratory carcinogen in rodents (*5*); and has been judged to pose a potential carcinogenic risk to humans (*1*).

The mutagenic and carcinogenic effects of *N*-nitrosamines, such as DMNA, and *N*-nitrosamides, such as *N*-methyl-*N*-nitrosourea (NMU), have been related to the degree of alkylation at the O^6-position of guanine in target tissue DNA and the ability of the tissue to remove this lesion (*6–9*). NMU methylates DNA at several sites, including O^6-guanine and *N*7-guanine (*10, 11*). The 7-methylguanine lesion is repaired slowly (half-life, 40 to 50 hours) (*11*), while O^6-methylguanine is repaired by O^6-alkylguanine DNA alkyltransferase at a much faster rate. Thus over short periods decreases in the ratio of O^6-methylguanine to 7-methylguanine can be used as a measure of O^6-methylguanine repair.

We investigated the repair of promutagenic O^6-methylguanine lesions induced by NMU in human bronchial fibroblasts. After a 1-hour exposure of these cells to 200 μM NMU, ratios of O^6-methylguanine to 7-methylguanine of between 0.029 and 0.054 were found (Table 1) (*12*). After 5 hours of posttreatment incubation of the cells in NMU-free medium, the ratio decreased from a mean of 0.041 to 0.009. Therefore only 20 percent of the O^6-methylguanine observed immediately after treatment with NMU for 1 hour remained in DNA 5 hours later. This observation is consistent with the kinetics of O^6-methylguanine and 7-methylguanine repair previously observed in human skin fibroblasts (*13*). However, when NMU-treated cells were incubated in the presence of 100 or 300 μM formaldehyde, a significantly lower rate of O^6-methylguanine removal was observed in three independent experiments. The removal of 7-methylguanine was not affected by formaldehyde and occurred at a rate (5 to 10 percent between hours 1 and 6) similar to that reported previously (*13*).

Formaldehyde could inhibit the repair of O^6-alkylguanine DNA alkyltransferase by several mechanisms, including steric hindrance of this DNA repair enzyme due to cross-links between DNA and DNA-associated proteins formed by formaldehyde. Because O^6-alkylguanine DNA alkyltransferase has a cysteine at its active site (*9*) and aldehydes readily bind to cysteine, formaldehyde and aldehydes formed by lipid peroxidation caused by formaldehyde-induced membrane damage could inhibit removal of O^6-methylguanine by binding to the active site of the alkyltransferase. This hypothesis is supported by recent observations that exposure of human bronchial epithelial and fibroblastic cells in vitro to formaldehyde or other thiol-binding aldehydes, such as acrolein and 4-hydroxy alkenals, preferentially inhibit O^6-alkylguanine DNA alkyltransferase activity when compared to that of another DNA repair enzyme, uracil DNA glycosylase (*14*).

To further study the pathobiological consequences of formaldehyde exposure and inhibition of O^6-methylguanine repair, we investigated the cytotoxic and mutagenic effects of formaldehyde and NMU separately and in combination. Cytotoxicity was determined by measuring the colony-forming efficiency of human fibroblasts that had been exposed to 100 to 800 μM NMU for 1 hour or 50 to 175 μM formaldehyde for 5 hours or to both as in the previous experiments, albeit at clonal rather than mass culture density. Compared to cells growing in logarithmic phase at clonal density, nearly confluent and slowly growing cultures

Table 1. Effect of formaldehyde on the removal of O^6-methylguanine from DNA of human fibroblasts exposed to NMU. Confluent human bronchial fibroblasts (HBF 357) were used to minimize the effects of cell cycle variations on growing cells. Cells were grown to confluence (15×10^6 to 16×10^6 cells per flask) in GDS medium (*18*). Sixteen flasks (four per determination) were incubated with tritiated NMU (1.6 Ci/mmol, New England Nuclear) at a concentration of 200 μM in 0.1 percent (by volume) dimethyl sulfoxide in phosphate-buffered saline (PBS; 4.4 ml per flask) for 1 hour at 37°C. After 1 hour the cells were washed once with PBS and then incubated in the presence or absence of formaldehyde [100 or 300 μM in 20 ml of EGM:LHC-1 (1:1) medium (*18*) per flask] for an additional 5 hours. At indicated times the cells were washed twice with PBS, harvested by trypsinization, and frozen as pellets at −70°C. DNA was purified by treatment with proteinase K and ribonuclease followed by phenol extraction and precipitation in ethanol by standard procedures. The levels of methylated DNA purines were analyzed by hydrolyzing the DNA in 0.1*M* HCl for 1 hour followed by separation by high-performance liquid chromatography (*19*). Values are ratios of O^6-methylguanine to 7-methylguanine.

Treatment	Experiment			Mean (percent)
	1	2	3	
NMU	0.054	0.029	0.039	0.041 (100)
NMU + fresh medium	0.008	0.006	0.012	0.009* (21)
NMU + 100 μM formaldehyde	0.014	0.014	0.015	0.014* (35)
NMU + 300 μM formaldehyde	0.024	0.019	0.019	0.021* (52)

*Each ratio is significantly different from other ratios ($P \leq 0.05$, Freidman test and Student's *t*-test).