Morphological, Genetic and Functional Variability of a T-Cell Hybridoma Line

(T-cell hybridoma / variability / chromosome number / reactivity / TCR / mouse)

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Abstract. The variability in the morphology, modal number of chromosomes, TCR expression and functional reactivity of a CII-specific T-cell hybridoma at continuous subcultivation have been investigated. As the number of passages increased, besides the oval semiadherent cells (normal phenotype), fibroblast-like cells (transformed phenotype) were also observed. The two cell subpopulations differed in their karyotype characteristic, as well as in their functional reactivity. The cell population with a normal phenotype was characterized by a tetramodal number of chromosomes (30, 40, 48 and 70) and trisomies of chromosomes 6 and 14, while the cell population with a transformed phenotype was characterized by a trimodal number of chromosomes (11, 68 and 74) and trisomy of chromosome 12. A nullisomy of sex chromosomes was established in both types of cells. In the initial passages of subcultivation, 73.04% of the cells with a normal morphological phenotype expressed TCR-CD3 complexes on their surface and possessed high functional reactivity. After a two-week subcultivation, the values of these indices went down considerably: 46.11% of the cells expressed functional TCR-CD3 complexes, as a result of which their functional reactivity decreased. Only 2.71% of the cells with a transformed morphological phenotype expressed functional TCR-CD3 complexes on their surface. In these cells, a total loss of reactivity towards the specific antigens was established. The achieved results show that at continuous subcultivation the T-cell hybridomas are unstable, and with the increase in the number of passages there appear chromosome rearrangements, leading to loss of their functional reactivity.

Received January 7, 2003. Accepted February 17, 2003.

This work was partly supported by the Swedish Strategic Foundation, Inflammation Research Network.

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Abbreviations: Ab – antibodies, Ag – antigen, APCs – antigen-presenting cells, BSA – bovine serum albumin, CII – type II collagen, CFA – complete Freund's adjuvant, CIA – collagen-induced arthritis, Gal – β-D-galactopyranose, Hyl – (5R)-5-hydroxy-L-lysine, IL – interleukin, TCR – T-cell receptor.

Hybridomas have general application in the contemporary biological and medical studies, as well as in biotechnologies for production of different biological and pharmaceutical products. The maintenance of their genetic stability and functional reactivity is of great significance. Quite often, the hybridoma cell lines reduce or totally lose their ability to produce monoclonal antibodies (Altshuler et al., 1986; Tharakan and Chau, 1986; Frame and Hu, 1989; Heath et al., 1990; Ozturk and Palsson, 1990a, b) or cytokines. In most of the cases, this is due to structural rearrangements in their genome. Predominantly, it is due to loss of chromosomes in which the genes expressing separate immunoglobulin chains are located (Swan et al., 1979; D'Eustachio et al., 1980, 1981; Köhler, 1980; Meo et al., 1980; Gardner et al., 1985; Nowak, 1985).

T-cell hybridomas are used mainly for investigation of the T-helper (Th) cell activity: determination of the T-cell functional potentials, establishment of T-cell receptor (TCR) peptide specificities, following infections or immunizations, clarification of the mechanisms of antigen processing/epitope selection (Hurwitz et al., 1984; White et al., 1989; Michaëlsson et al., 1994; Malmström et al., 1996; Corthay et al., 1998; Harrington et al., 1998; Latek and Unanue, 1999; Nagel et al., 2000; Wellner et al., 2000; Nakajima et al., 2001; Qadri and Ward, 2001; Surman et al., 2001; Mansour et al., 2002).

Many scientific laboratories generate specific, in relation to different antigens, T-cell hybridomas, which are used for different immunological studies. The loss of such cell lines or of their reactivity makes the reproduction of certain experiments and results impossible.

In the current study, we analyse the changes that occur after several passages of *in vitro* subcultivation of a type II collagen (CII)-specific T-cell hybridoma, which is used in the CIA experimental models (Michaëlsson et al., 1994; Malmström et al., 1996;

Corthay et al., 1998; Wellner et al., 2000; Bäcklund et al., 2002). Our study was aimed at establishing the relationship between chromosome rearrangements in the karyotype, TCR expression, and the changes that occur in the morphology and functional reactivity of the studied T-cell hybridoma.

The achieved results demonstrate that in conditions of continuous cultivation the T-cell hybridomas are unstable. As the number of passages increases, there occur structural rearrangements in their genome, leading to loss of their functional reactivity.

Material and Methods

Cells and antigens

Dulbecco's minimum essential medium (DMEM), foetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL, Life Technologies Ltd. (Paisley, UK).

The T-cell hybridoma HCQ.10 has been characterized (Corthay et al., 1998). This hybridoma is generated by polyethylene glycol-induced fusion of lymph node cells from C3H.Q mice (immunized 10 days previously with rat CII/CFA) with TCRα^-/β^- BW5147 thymoma cells (White et al., 1989). HCQ.10 specifically recognizes CII and a posttranslationally modified CII (256–270) peptide with Gal-Hyl at position 264 presented by H-2A^q expressing antigen-presenting cells (APCs). Cells were cultured routinely in 75 cm^2 culture flasks in DMEM supplemented with Glutamax-II, 10% FBS, 100 U penicillin and 100 µg/ml streptomycin in a Heraeus incubator at 37˚C with 5% CO_2 in air and high humidity.

Spleen cells from C3H.Q mice were used as APCs.

Rat CII was prepared from Swarm chondrosarcoma (Smith et al., 1975) by limited pepsin digestion (Miller, 1972; Andersson and Holmdahl, 1990). The glycopeptide assembly of the synthetic peptide CII (256–270) has been described (Broddefalk et al., 1996). Both antigens were stored lyophilized and dissolved in 0.1 M acetic acid at 4˚C.

Karyotype analysis

Metaphase spreads were prepared according to the standard method of Rothfels & Siminovitch (1958). Cells were incubated with 0.2 µg/ml colcemid for 2 h at 37˚C. Subsequently, the cells were treated with 75 mM KCl solution for 15 min at room temperature and after fixation and rinsing in methanol/acetic acid (3 : 1), the cell suspension was pipetted onto microscope slides and allowed to dry using the air-dried technique. G-banding was performed according to the technique described by Seabright (1971). Chromosomes were stained with 10% Giemsa solution in phosphate buffer (pH = 7.2) for 10 min.

For determination of the modal chromosome number and chromosome alterations, 100 metaphases were analysed. Chromosomes were identified on the basis of their banding patterns according to the Committee on Standardized Genetic Nomenclature for Mice (1972).

Flow cytometry

TCR expression levels were analysed by flow cytometry analysis using PE-conjugated anti-mouse TCRβ chain (clone H57-597) antibodies and FITC-conjugated anti-mouse CD3ε (clone 145-2C11) antibodies (PharMingen, Los Angeles, CA). Cells were washed and resuspended in PBS supplemented with 0.5% (w/v) BSA (Sigma, St Louis, MO) and 0.01% (w/v) sodium azide (staining buffer). Staining with Ab was performed for 15 min at 4˚C. The cells were then washed twice with staining buffer and analysed immediately. For flow cytometry analysis, a typical forward and side scatter gate was set to exclude dead cells and aggregates. In total, 10^4 events in the gate were collected and analysed using a FACSort (Becton Dickinson, San Jose, CA) equipped with CellQuest software.

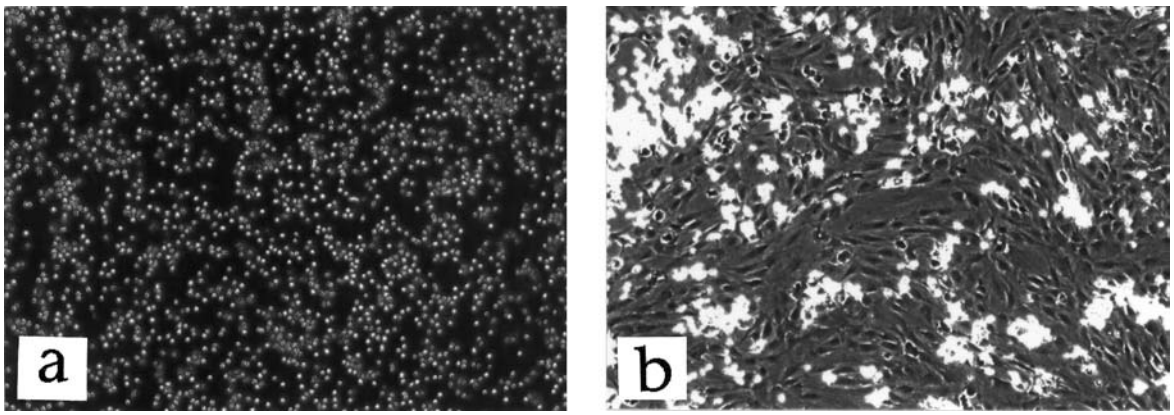

Fig. 1. A phase contrast micrograph of the HCQ.10 T-cell hybridoma with two morphologically distinct subpopulations: **a** – semiadherent cells with oval shape, **b** – adherent fibroblast-like cells. (Magnification 40x)

Assay for determination of T-cell hybridoma response

Activation of H-2A^q-restricted CII-specific HCQ.10 T-cell hybridoma *in vitro* with antigen (Ag) and syngeneic APCs resulted in secretion of interleukin-2 (IL-2) in a dose-dependent fashion.

For the measurement of T-cell hybridoma reactivity, 5 x 10^4 T-cell hybridomas were incubated with 5 x 10^5 syngeneic, irradiated (1500 rad) spleen cells (APCs) in the presence of titrated concentrations of Ag in a volume of 200 µl in 96-well flat-bottom plates (Nunc, Roskilde, Denmark). After 24 h, the contents of IL-2 in the culture supernatant was measured by sandwich ELISA (purified rat anti-mouse IL-2 as capturing antibodies (Ab) and the biotin rat anti-mouse IL-2 as detecting Ab, both from PharMingen, Los Angeles, CA) using the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) system based on the time-resolved fluoroimmunoassay technique with europium-labelled streptavidin (Wallac, Turku, Finland). Recombinant mouse IL-2 served as a positive control and standard.

Results

Morphological analysis

HCQ.10 cells as well as most T-cell hybridomas are semiadherent cells with a round/polygonal/oval shape. These normal morphological characteristics are observed during the first 1–2 weeks of the HCQ.10 subcultivation, after their defreezing (Fig. 1a). The same morphology is also characteristic for the fusion partner (BW5147), used for the generation of the HCQ.10 hybridoma cell line.

During the second week of subcultivation, two morphologically distinct subpopulations might have been clearly distinguished: oval semiadherent cells of ”normal” type, with high proliferative activity, and firmly attached fibroblast-like cells, which formed a contact-inhibited monolayer (Fig. 1b). The two cell subpopulations were separated and afterwards they were separately subcultivated.

At subcloning of the ”normal” type cell population, similar heterogeneity was obtained again after a two-week subcultivation of the newly received clones (data not shown). The population of cells with the “transformed” phenotype (adherent fibroblast-like cells) was cultivated for a period of two months (10 passages). No restoration of the normal morphological phenotype was observed.

The two morphological cell types were subject to karyotype analysis, as well as subject to tests for functional reactivity and expression of surface TCR markers.

Karyotype analysis

The karyotype of the HCQ.10 T-cell hybridoma was studied in passages 15 (for the cells with the normal morphological phenotype) and 10 (for the cells with the transformed morphological phenotype). Both morphological phenotypes of cells are characterized by their high karyotypic heterogeneity.

At the 15th passage of cultivation of ”normal” HCQ.10 cells, quite a wide range of variations in the number of chromosomes, from 13 to 88, was observed in separate cells, and for these cells a tetramodal number of chromosomes (30, 40, 48 and 70) was established (Fig. 2a). The karyotypic analysis of the “transformed” HCQ.10 cells showed a trimodal number of 11, 68 and 74 chromosomes (range: 7–89) (Fig. 2b). The BW5147 cell line, used for fusion, contained a near-diploid chromosome number of 38 chromosomes, with a range of 8–40 chromosomes (Fig. 2c).

Cytogenetic analysis showed that the HCQ.10 cells with the transformed morphological phenotype contain only acrocentric chromosomes, while in 74% of studied metaphase plates of the HCQ.10 cells with the normal morphological phenotype, one metacentric chromosome was found (Fig. 3a). Two metacentric chromosomes were observed in 82% of the studied metaphases in the BW5147 cell line (Fig. 3b). Sixty-seven percent of the BW5147 cells also contained one chromosome with secondary constriction, which might be used as a marker (Fig. 3c).

The analysis of G-banded karyotypes indicated high variability in the chromosome content of the separate cell lines.

In HCQ.10 cells with the normal morphological phenotype, trisomies of chromosome 6 and chromosome 14 were found (Table 1). Trisomies of the same chromosomes were also found in the BW5147 cells, which were used as a fusion partner. In all studied metaphase plates of both cell lines, monosomy of chromosome 13 and loss of the sex pair were established.

In HCQ.10 cells with the transformed morphological phenotype, trisomy of chromosome 12 in 66% of the studied metaphases (Table 1) and monosomy of chromosomes 2 and 5 in all metaphase plates were found. Trisomies of chromosomes 6 and 14 of these cells were not observed. Nullisomy of the sex chromosomes was found again.

No structural alterations were detected by the used methods.

TCR expression and functional reactivity

Cell surface expression of TCR-CD3 complexes was examined by flow cytometry using staining with anti-TCR β-chain and anti-CD3e antibodies.

After a one-week subcultivation of the HCQ.10 cell line (two passages), 73.04% of the cells expressed functional TCR-CD3 complexes on their surface, and

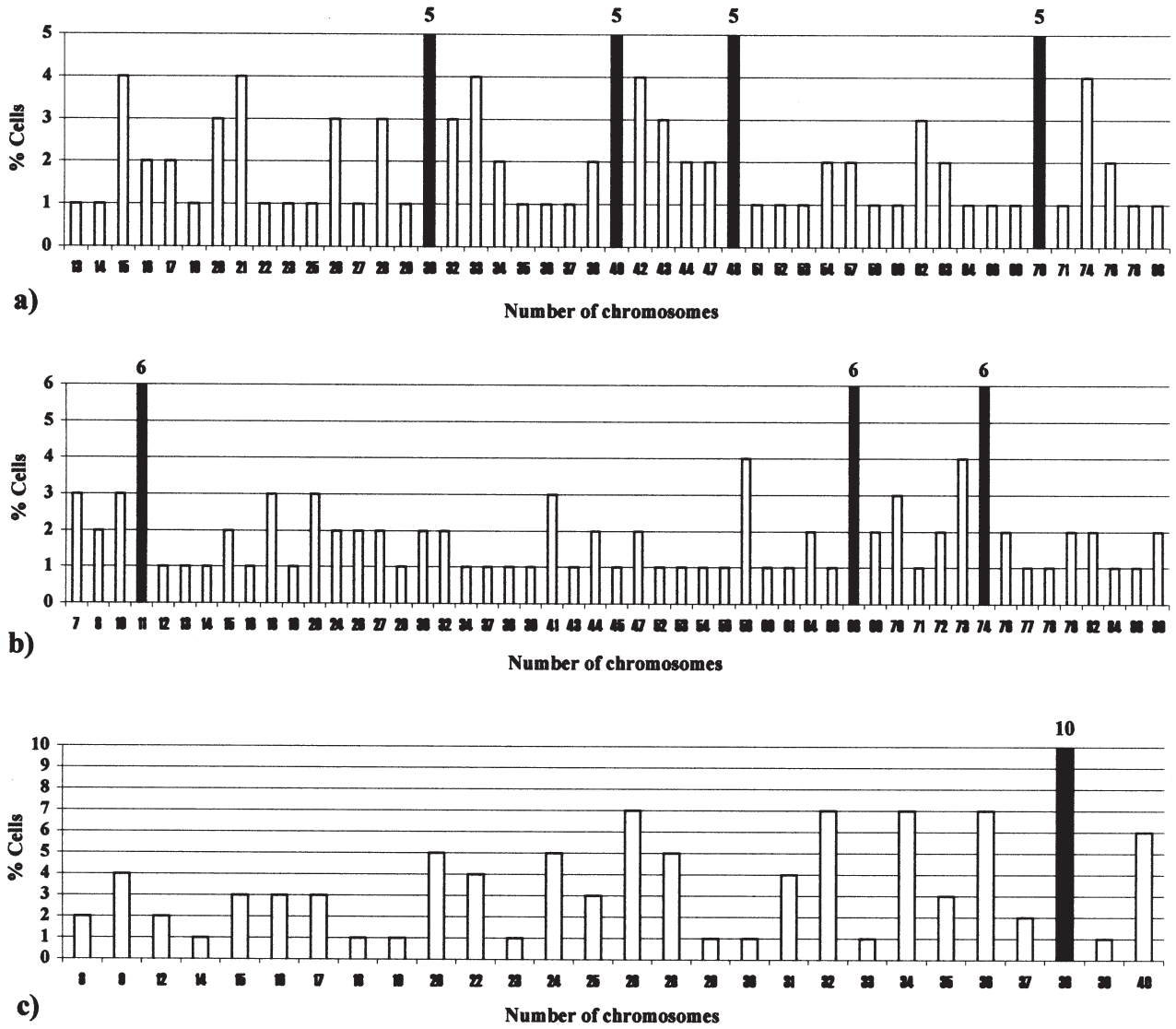

Fig. 2. Chromosomal distribution and modal number of chromosomes (filled bars) of «normal» HCQ.10 cells at 15th passage (**a**), «transformed» HCQ.10 cells at 10th passage (**b**) and BW5147 cells (**c**).

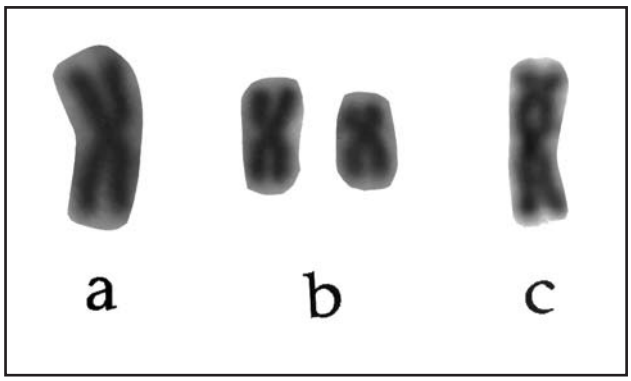

Fig. 3. Metacentric chromosomes in «normal» HCQ.10 cells (**a**) and BW5147 cells (**b, c**). The chromosome with secondary constriction (**c**) might be used as a marker for the BW5147 cell line.

24.35% of the cells expressed only CD3 molecules (Fig. 4a). At subcultivation of HCQ.10 hybridoma cells for a period of two weeks (five passages), the percentage of cells expressing functional TCR-CD3 complexes decreased to 46.11% (Fig. 4b). In that case, 8.89% of the cells expressed only CD3 molecules, while 43.89% were double negative – they did not express either TCR (β-chain), or CD3 molecules.

In the tenth passage of subcultivation of HCQ.10 cells with the transformed morphological phenotype, only 2.71% of the cells still possessed the functional TCR-CD3 complex, 79.67% of all cells expressed only CD3 molecules, and 17.62% were double negative (Fig. 4c). The lack of functional TCR (TCR$\alpha^-\beta^-$) in the BW5147 cells, which were used as a fusion partner at generation of the HCQ.10 hybridoma cell line, was confirmed by the held FACS analysis (Fig. 4d).

Table 1. Numerical abnormalities of G-banded chromosomes found in the HCQ.10 T-cell hybridoma and BW5147 cell line

Chromosome	HCQ.10 cells		BW5147 cells
	normal phenotype	transformed phenotype	
2	–	monosomy (100 %)	–
5	–	monosomy (100 %)	–
6	trisomy (70 %)	–	trisomy (57 %)
12	–	trisomy (66 %)	–
13	monosomy (100 %)	–	monosomy (100 %)
14	trisomy (66 %)	–	trisomy (62 %)
X	nullisomy (100 %)	nullisomy (100 %)	nullisomy (100 %)
Y	nullisomy (100 %)	nullisomy (100 %)	nullisomy (100 %)

HCQ.10 T-cell hybridoma is H-2A^q-restricted and specifically recognized rat CII and its synthetic glycosylated analogue CII (256–270) with Gal-Hyl at position 264 (Corthay et al., 1998). These cells secrete IL-2 in a dose-dependent fashion when cultured together with Ag and spleen cells as APCs. The reactivity of HCQ.10 T-cell hybridoma towards rat CII and synthetic Gal-Hyl-264 CII (256–270) peptide was tested using ELISA detection of IL-2 production.

The reactivity data of HCQ.10 T-cell hybridoma correlate with the expression of TCR-CD3 complexes on their surface. When stimulating the HCQ.10 cells, subcultivated for a week with the antigens that they specifically recognize (rat CII and Gal-Hyl-264 CII (256–270) peptide) and APCs, a high level of IL-2 secretion was detected. The hybridoma cells responded well to both rat CII and Gal-Hyl-264 CII (256–270) peptide (Fig. 5a,b). At subcultivation of HCQ.10 cells for two weeks (when only 46.11% of all cells expressed TCR-CD3 complexes) the production of IL-2 and the hybridoma cells' reactivity, respectively, considerably decreased (Fig. 5). It was not surprising that, for the HCQ.10 cells with the transformed morphological phenotype, there was established a total loss of functional reactivity (Fig. 5), having in consideration the lack of expression of TCR-CD3 complexes on their surface.

Discussion

Although the biological functions and mechanisms at *in vivo* conditions are much more complicated, *in*

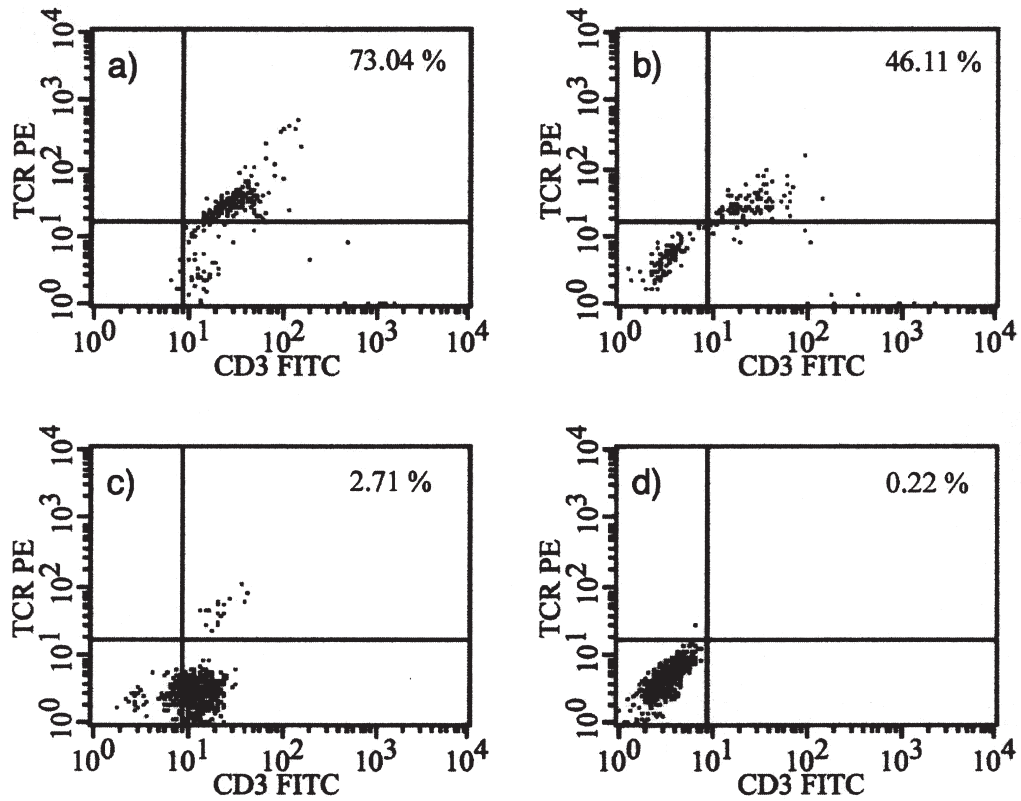

Fig. 4. Expression of TCR-CD3 complexes on the surface of «normal» HCQ.10 T cells after 1 week of subcultivation (**a**), «normal» HCQ.10 T cells after 2 weeks of subcultivation (**b**), «transformed» HCQ.10 T cells at the 10th passage (**c**) and BW5147 cells (**d**). The percentage of double-positive cells is indicated.

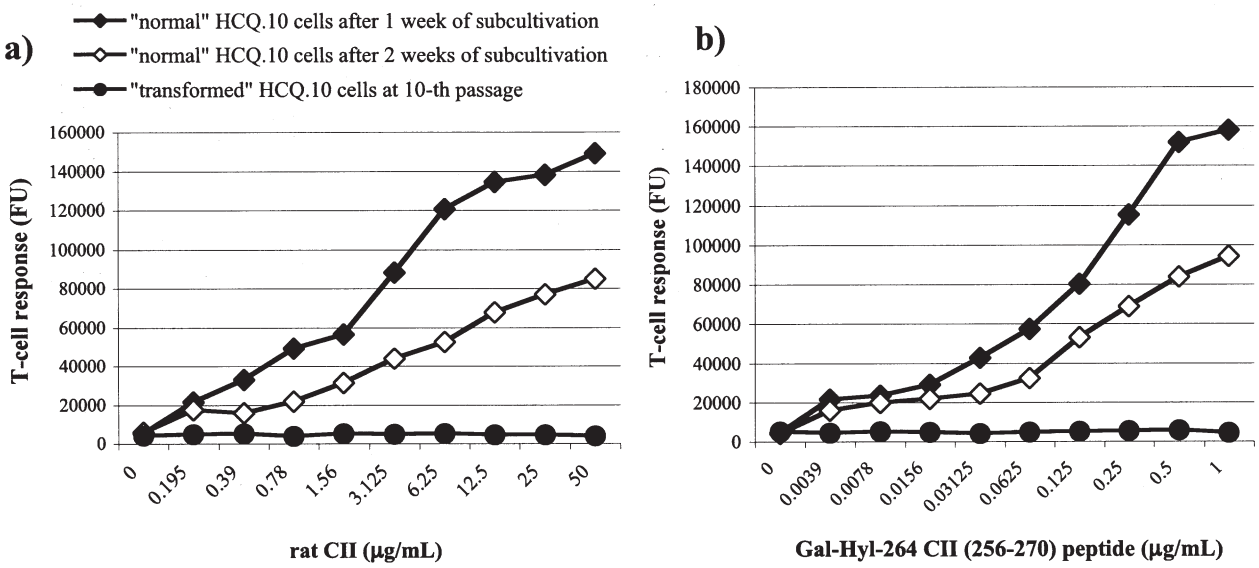

Fig. 5. HCQ.10 T-cell hybridoma activation in response to rat CII (**a**) and Gal-Hyl-264 CII (256-270) peptide (**b**). The results are presented as mean values of triplicates (FU – fluorescence units).

vitro research works are a significant stage in the investigations of mechanisms of immunity answers and autoimmune diseases.

It is well known that T cells are exquisitely sensitive to antigenic stimulation, since they can proliferate and produce cytokines in response to APCs, displaying as few as one to a few hundreds of specific peptide-MHC complexes (Demotz et al., 1990; Harding and Unanue, 1990; Valitutti et al., 1995).

After fusion most murine-murine hybridomas are unstable in their ability to produce specific monoclonal Ab or cytokines. The decrease or loss of their functional reactivity is attributed to chromosome rearrangements and chromosome losses. The total chromosome number in hybridomas frequently does not correspond to the sum of chromosomes from both fused cells (Rodova et al., 1985; Volgareva, 1985; Kontsek et al., 1988; Zhil'tsova et al., 1989; Wollweber et al., 2000).

In this study the morphological, karyotypic and functional variability as well as exposure of cell surface markers of a CII-specific T-cell hybridoma (HCQ.10) were examined.

The two different morphological phenotypes of cell populations, observed during the subcultivation of the HCQ.10 hybridoma cell line, differ in their karyologic characteristic, as well as in their expression of TCR-CD3 complexes and functional reactivity. The cell population with the normal morphological phenotype is characterized by a tetramodal chromosome number, while the one with the transformed morphological phenotype – by a trimodal number of chromosomes (Fig. 2). The established trisomies of chromosomes 6 and 14 in HCQ.10 cells with the normal morphological phenotype (Table 1) correlate with the expression of TCR-CD3 complexes (Fig. 4) and the functional reactivity of these cells (Fig. 5), having in consideration the fact that the genes encoding the TCR α- and β-chains are located in chromosome 14 and chromosome 6, respectively. The functional reactivity of hybridoma cells depends exactly on the increased number of copies of these chromosomes and the expression of functional TCRs. In BW5147 cells, used as a fusion partner, trisomies of these chromosomes were also found, but the cells did not express functional TCRs (Fig. 4d; White et al., 1989). On the other side, the established monosomy of chromosome 13 in the normal HCQ.10 cells and BW5147 cells correlate with the fact that these cells do not express TCR with the γ-chain, but the gene coding the γ-chain of TCR is located in chromosome 13.

Fabris et al. (2001) reported about a trisomy of chromosome 14 at a cytogenetic analysis of the LBL cell line, established from T-cell lymphoid leukaemia (LB), and showed that trisomy of this chromosome plays a crucial role in the development of LB lymphoma.

In the HCQ.10 cells with the transformed morphological phenotype a trisomy was found only on chromosome 12, where the gene encoding immunoglobulin heavy chains is located (D'Eustachio et al., 1980; Meo et al., 1980). According to Wollweber et al. (2000), chromosome 12 plays an important role in the survival and stability of the mouse-mouse hybridomas. Most probably, this chromosome is also connected with the expression of adhesive molecules, having in consideration the morphological phenotype of the transformed HCQ.10 cells (Fig. 1b).

The loss of the sex chromosomes is a natural phenomenon in the process of continuous cultivation of animal and human cell lines. It might take place at the earliest, as well as at some later stages of cell line stabilization (Mamaeva, 1996).

The rearrangements and stabilization of the hybridoma genome occurred during the initial first few cell divisions (Wollweber et al., 2000). Similar results were obtained by Kontsek et al. (1988) during the first two months to a year of long-term culture by means of trypsin-Giemsa staining. According to Castillo et al. (1994), most murine hybridomas, selected after cloning and subcloning following fusion, have remained highly stable in respect of growth and antibody production.

The results achieved by us showed that at continuous subcultivation of T-cell hybridomas and with the increase in the number of passages, considerable chromosome rearrangements occur, which lead to loss of the functional reactivity of these cells on the one side, and to adaptation to growth and development at *in vitro* conditions, on the other. Therefore, the T-cell hybridomas are unstable and they demonstrate high functional reactivity during the first 2–3 weeks. For this reason, they should not be cultivated as continuous cell lines, but they should be subcloned and freezed every 2–3 weeks. They are characterized by their high karyotypic heterogeneity and their continuous subcultivation leads to stabilization of their karyotype, most often with a near-diploid modal number of chromosomes (unpublished data), but at the same time, it leads to the loss of TCR expression and of their reactivity, respectively.

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