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Immune defects in families and patients with xeroderma pigmentosum and trichothiodystrophy

E. MARIANI*†, A. FACCHINI*†, M. C. HONORATI*†, E. LALLI§, E. BERARDESCA¶, P. GHETTI‡, S. MARINONI‖, F. NUZZO**, G. C. B. ASTALDI RICOTTI** & M. STEFANINI** *Laboratorio di Immunologia e Genetica, Istituto di Ricerca Codivilla Putti, I.O.R., Bologna, †Istituto di Clinica Medica e Gastroenterologia, ‡Istituto di Clinica Dermatologica, Università di Bologna, §Istituto di Citomorfologia C.N.R., Chieti, ¶Clinica Dermatologica, Policlinico S. Matteo, Pavia, ‖Istituto per l'Infanzia, Trieste, and **Istituto di Genetica Biochimica ed Evoluzionistica C.N.R., Pavia, Italy

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SUMMARY

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by photosensitivity, a high incidence of cancer in sun-exposed portions of the skin and a reduced capacity to repair the u.v.-induced DNA damage. One of the XP mutations (XP-D) has also been identified in patients affected by trichothiodystrophy (TTD), a rare autosomal recessive disease characterized by brittle hair, mental and physical retardation, peculiar face and ichthyosis. However, in these patients there is no evidence of increased skin tumour incidence. Since an impairment of cell-mediated immunity has been proposed as a co-factor in the cancer proneness of XP patients, we investigated the involvement of immune defect(s) in five XP patients, five TTD patients, their parents, and 24 TTD relatives. We evaluated the phenotype of circulating lymphocytes, natural killer (NK) cell lytic activity, target cell binding of NK cells at single cell level and the effect of interferons (IFN) α and β on NK cell activity. The relative proportion of CD3$^+$ and CD4$^+$ circulating lymphocytes was reduced in XP but not in TTD patients. NK cell lytic activity was decreased in XP patients and their mothers, but their fathers showed normal lytic activity. NK activity varied among TTD families: four out of five patients and their relatives presented low NK cell activity, and one family was normal. In TTD family members, NK activity increased after incubation with IFN-α or IFN-β, but never reached normal values. In contrast, in XP patients and their mothers, the defect was almost completely corrected after *in vitro* incubation with IFN-α or IFN-β. Our study indicates impaired NK lytic activity in the majority of TTD and XP patients and that this defect is present also in members of their families. In addition, XP patients present a low number of circulating T cells. These multiple abnormalities, together with DNA repair defects, could be related to the increased cancer risk in XP patients.

Keywords xeroderma pigmentosum trichothiodystrophy natural killer cells cancer proneness immunodeficiency

INTRODUCTION

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by photosensitivity, a high incidence of cancer in sun-exposed portions of the skin and a reduced capacity to repair the u.v.-induced DNA damage. By complementation analysis, eight complementation groups have been identified to date: seven groups (termed XP-A to XP-G) are defective in the excision repair mechanism and one (termed XP variant, XP-V) in the post-replication repair [for reviews see 1,2]. Since in most patients the degree of the cellular u.v. hypersensitivity correlates with the severity of clinical symptoms, XP is considered the best example of the relationship between defective DNA repair and cancer proneness.

In 1986, the same genetic defect present in XP group D was described by Stefanini *et al.* [3] in four Italian patients showing clinical symptoms diagnostic for trichothiodystrophy (TTD), together with photosensitivity. TTD is a rare autosomal recessive disease characterized by brittle hair, mental and physical retardation, peculiar face and ichthyosis. Photosensitivity is reported in about 20% of cases [reviewed in 4].

Cellular and genetic studies in several patients from different countries demonstrated that normal or enhanced u.v. sensitivity is associated with TTD and that in the repair-deficient TTD cell strains the XP-D mutation is present [5–10]. However, severe skin lesions and tumours typical of XP have not been reported in any TTD patient. Recently, a decreased natural killer (NK)

Correspondence: Dr Erminia Mariani, Laboratorio di Immunologia e Genetica, Istituto di Ricerca Codivilla Putti, Via di Barbiano 1/10, 40136 Bologna, Italy.

Table 1. Clinical features and DNA repair data of patients with xeroderma pigmentosum (XP) and trichothiodystrophy (TTD)

Patient	Age (years)	Sex	Skin cancer	Neurologic anomalies	UDS (% normal)	Complementation group
XP5	21	M	+	−	15	C
XP12	7	M	+	−	20	C
XP13	16	M	+	−	19	C
XP17	33	M	+	+	5	D
XP19	4	M	−	−	18	C
TTD1	22	F	−	+	11	D
TTD2*	13	F	−	+	8	D
TTD3*	11	F	−	+	10	D
TTD6	2	F	−	+	35	D
TTD7	2	F	−	+	14	D

UDS, unscheduled DNA synthesis.
* Sisters.

activity was described in five XP patients but not in the TTD patient [11,12]. The identification of the factor(s) involved in the different incidence of tumours in XP and TTD may offer crucial information on the mechanisms underlying cutaneous carcinogenesis.

Here we have investigated the involvement of the immune defect(s) which may be related to the cancer proneness of XP in five XP patients, five TTD patients, their parents and 24 TTD relatives.

SUBJECTS AND METHODS

Patients and controls

The study was performed on five patients (from five families) with XP and five patients (from four families) with TTD. The main clinical features and the DNA repair data of the patients are shown in Table 1. All were Caucasian Italians and had been protected from sun exposure since early childhood. Together with photosensitivity, the TTD patients showed only some freckles on the face, whereas the XP patients showed typical dyspigmentary and dyskeratotic cutaneous alterations. Cutaneous tumours (basal cell carcinoma and squamous cell carcinoma) were absent in TTD patients but present in all but the youngest XP patient. Impaired capacity to repair the u.v.-induced DNA damage was observed in fibroblasts and lymphocytes from all the patients, with DNA repair synthesis (unscheduled DNA synthesis (UDS)) levels ranging between 5% and 35% of normal. By complementation analysis, the five TTD patients were classified into XP group D, four XP patients were classified into XP group C and one into group D [3,9, and unpublished data].

The investigation was extended to 44 phenotypically normal members of the patients' families (23 women, 21 men); 20 were obligate heterozygotes (the parents of the five XP patients, the parents of the five TTD patients and of a dead TTD patient reported as TTD4 [3,9]); 24 were facultative heterozygotes (two brothers, nine aunts, six uncles and seven grandparents) belonging to the four families of patients TTD1, 2, 3, 4 and 6. At the time of the examination, the age range of the patients' parents was 21–62 years. DNA repair investigations demonstrated normal response to u.v. irradiation in the fibroblasts from the XP and TTD patients' parents [9, and our unpublished observations]. Only parents and grandparents of TTD6 patient and the father of TTD4 patient were smokers, and one out of seven grandparents (grandmother of TTD6 patient) had diabetes. Twenty-nine matched normal healthy controls over 20 years of age and at least five matched normal healthy controls for different ages (2 years, 4 years, 11–13 years, 16 years) were selected according to the biochemical and clinical parameters [13]. Patients, parents and relatives gave their informed consent to enrol in the present study, which was approved by the local Ethical Committee.

Mononuclear cell preparation

Blood was collected in heparin (Liquemin, Roche, Switzerland) by venepuncture of the cubital vein. Total white blood cell (WBC) counts and leucocyte differentials were obtained on all the samples.

Mononuclear cells (MNC) were isolated by conventional density gradient centrifugation, washed twice in RPMI 1640 medium + 10% heat-inactivated fetal calf serum (FCS) (Gibco, Grand Island, NY) and were used for the following assays.

MoAbs and immunofluorescence staining

The distribution of lymphocyte phenotypes in each patient was determined by direct fluorescent staining using a panel of fluorochrome-conjugated (FITC and PE) MoAbs CD3/PE (Leu 4), CD4/FITC (Leu 3a), CD8/PE (Leu 2a), CD16/FITC (Leu 11c), HLA-DR/FITC (Becton Dickinson). Staining of lymphocytes with MoAbs was performed as previously reported [13,14].

Briefly, 2×10^5 lymphocytes were incubated with each MoAb in V-bottomed 96-well microtitre plates. After 30 min at 4°C, the lymphocytes were washed twice with RPMI 1640 complete medium, centrifuged for 10 min at 4°C and resuspended for analysis.

Flow cytometry

Flow cytometric analyses were performed with a FACStar Plus cell sorter (Becton Dickinson) and a Hewlett-Packard computer

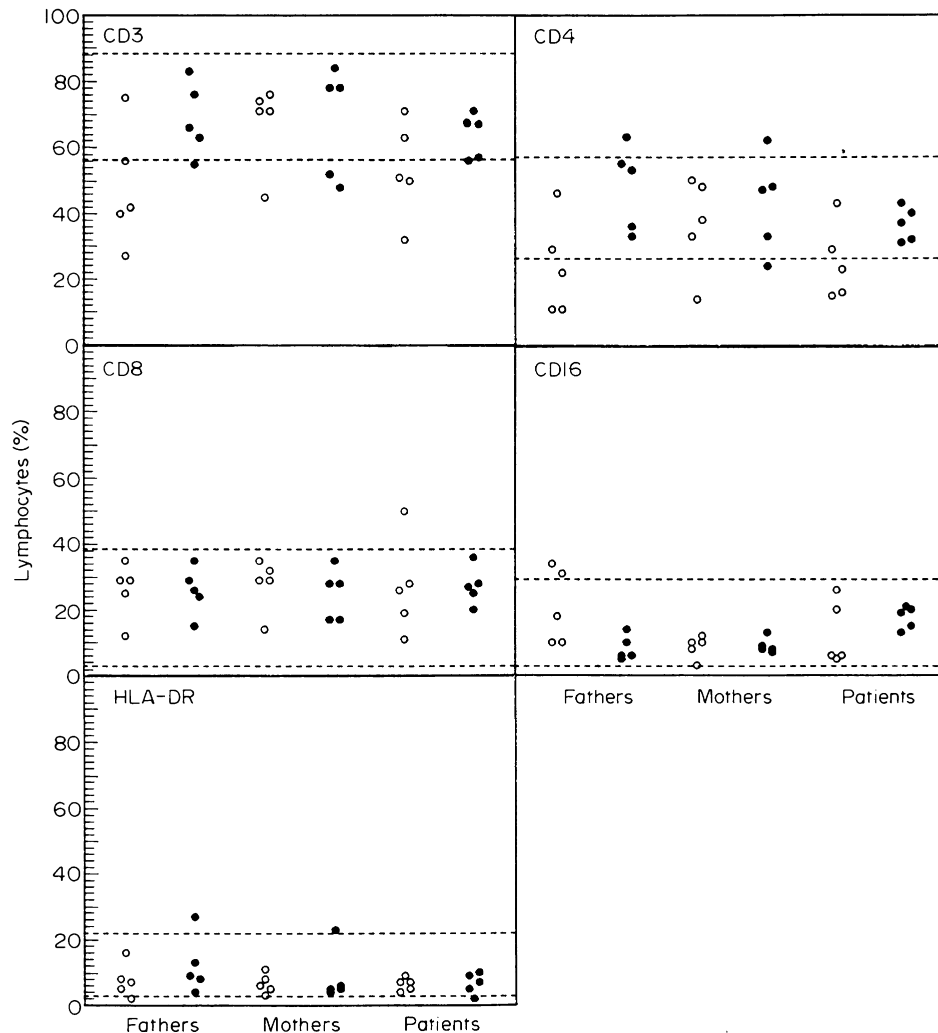

Fig. 1. Phenotypical distribution of circulating lymphocytes from XP (○) and TTD (●) families. The dashed lines represent respectively the upper and lower limits of the reference range derived from 29 matched healthy subjects.

was used for data storage. The FACStar was equipped with a 5 W argon ion laser (Spectra Physics), operating at 488 nm wavelength in order to excite both FITC and PE fluorochromes. Forward light scatter (FLS) (2°–15°) was collected with a 0·5% neutral density filter in front of the photodiode, while perpendicular light scatter (PLS) (75°–105°) was measured collecting 5% of the light through a 488 nm band pass filter with a photomultiplier tube (PMT). Furthermore, FITC green fluorescence was selected by a BP 530/30 nm filter and PE red fluorescence was selected by a BP 575/26 nm filter. Cell populations were selected by FLS and PLS using polygonal windows [15] which excluded dead cells and debris and 5×10^3 cells were analysed for each sample.

NK cell lytic activity

K562 tumour cell line was used as the target for cytotoxicity assays. Briefly, 2×10^6 K562 cells were incubated with 100 μCi of radioactive sodium chromate (NEN, Germany; specific activity 400–1200 Ci/g) with occasional shaking at 37°C in 5% CO_2 for 1 h. The tumour cells were then washed twice at 4°C in RPMI 1640 + 10% FCS and the cytotoxicity assays were performed in V-bottomed 96-well microtitre plates.

Varying numbers of effector lymphocytes (from 5×10^3 to 5×10^5) in 100 μl RPMI–HEPES with 10% FCS and 50 μl of ^{51}Cr-labelled target cells (5×10^3/well) were seeded in triplicate. After 4 h of incubation at 37°C in 5%CO_2, 75 μl of supernatant were harvested and ^{51}Cr release was determined with a gamma counter. Specific ^{51}Cr release was calculated according to the formula [16,17]

$$\frac{\text{Experimental} - \text{Spontaneous release}}{\text{Maximum} - \text{Spontaneous release}} \times 100$$

where spontaneous release represented the ^{51}Cr release from target cells with medium alone and the maximum release was the ^{51}Cr release from the target cells lysed by 1% Triton X-100.

Incubation with IFNs

Different aliquots of lymphocytes (1×10^6/ml) were cultured at 37°C in 5% CO_2 humidified atmosphere in RPMI 1640 + 10% FCS containing 1000 U/ml of recombinant IFN-α-2b (Intron-A, Shering Corporation) and IFN-β (Frone, Serono, Italy). After overnight incubation the lymphocytes were washed in medium alone, counted and used as effector cells in the cytotoxicity assay as described above.

Table 2. Absolute number of CD3$^+$ and CD4$^+$ circulating lymphocytes from XP and TTD families with decreased percentages

Family	Father	Mother	Patient (age/years)	
XP5				
CD3	864	835	812	(21)
CD4	453	260	373	
XP17				
CD3	900	1906*	889	(33)
CD4	300	1342*	384	
XP19				
CD3	889	1155*	1243	(4)
CD4	406	709*	622	
TTD7				
CD3	1535*	950	4651*	(2)
CD4	1202*	479	2611*	

Normal values are expressed as mean ± s.d. of positive cells/μl; normal adults healthy controls (> 20 year old): CD3, 1641 ± 369; CD4, 1010 ± 196; 4-year-old healthy controls: CD3, 2930 ± 426; CD4, 1784 ± 259; 2-year-old healthy controls: CD3, 3006 ± 1007; CD4, 1855 ± 516.

* The table also reports the values of mothers of XP17 and XP19 patients and of TTD7 father and TTD7 patient that showed percentage distributions within the range of the corresponding age controls.

Target cell binding at single cell level
The cell binding to target K562 was evaluated at single cell level according to Grimm & Bonavida [18]. Briefly, equal numbers of lymphocytes and target cells were incubated for 10 min at 30°C, then centrifuged for 5 min at room temperature to promote conjugate formation. After centrifugation the pellet was gently resuspended and a drop of this suspension was removed and diluted to determine the percentage of bound lymphocytes. This percentage was determined by counting the number of single lymphocytes bound to single target K562 cells per every 100 total lymphocytes. Tubes of target cells alone prepared in an identical manner served as a control.

Statistical analysis
Student's *t*-test was used to compare experimental data, which were expressed as mean ± s.e.m.

RESULTS

Analysis of the phenotype of circulating lymphocytes
The total WBC count (mean ± s.d. 4997 ± 1441/μl) and the differential lymphocyte count (mean ± s.d. 2257 ± 822/μl) in the parents and in XP and TTD patients over 20 years of age was similar to normal healthy matched controls (mean WBC ± s.d. 5250 ± 898/μl; mean lymphocytes ± s.d. 2204 ± 509/μl). The total WBC and differential lymphocyte counts of XP and TTD patients aged 2–16 were within the reference range derived from the corresponding age-matched controls.

The phenotypical distribution of peripheral blood lymphocytes was altered in XP patients but not in TTD patients. In particular the percentages (Fig. 1) and the absolute values (Table 2) of CD3$^+$ and CD4$^+$ circulating lymphocytes from XP subjects was lower in three patients (XP5, XP17, XP19), in their fathers and the mother of XP5. In contrast, the CD8$^+$, CD16$^+$ and HLA-DR$^+$ lymphocyte proportions were within normal range both for percentages (Fig. 1) and absolute numbers (data not shown). The phenotype of lymphocytes from TTD patients and their parents showed a normal distribution, with the exception of the TTD7 mother who had a slightly reduced proportion of CD3$^+$ and CD4$^+$ cells (Fig. 1 and Table 2). The complete phenotype of all the other 24 TTD family members analysed was within the normal range of the corresponding age-matched groups (data not shown). No influence of smoking or the presence of diabetes was observed on the parameters evaluated.

NK cell lytic activity of fresh lymphocytes
The NK cell lytic activity was reduced in XP patients, their mothers and all members of four out of five TTD families. Within each XP family NK activity was consistently lower in the affected patients and their mothers than in their fathers (Fig. 2a–e).

The mean NK activity of the five XP fathers (Fig. 2f) was within the reference range derived from 29 matched healthy subjects, but the mean response of the five XP patients and their mothers was significantly lower than normal ($P < 0.0003$ and $P < 0.0002$, respectively, for a target/effector cell ratio 1/50).

NK cell activity was also decreased in four out of five TTD families (Fig. 3b–e), although patient TTD1 and parents showed normal NK activity (Fig. 3a).

Two patients (TTD6 and TTD7) and their parents were tested for a second time 1 year later and their NK activity was still found to be reduced to the same extent (data not shown). The mean cytolytic activity from all TTD patients and their parents was significantly reduced ($P < 0.002$ for a target/effector cell ratio 1/50) compared with the reference range (Fig. 3f). Furthermore, the NK cell activity presented a uniform trend within each family when relatives from maternal and paternal branches were analysed (data not shown). In particular a reduced NK activity was found in six uncles, seven aunts and five grandparents of the TTD patients who had the lowest NK activity. The NK cell activity was normal only in the maternal grandmother of TTD6 patient. In contrast, a normal response was observed in four relatives (two brothers, one uncle and one aunt) of patient TTD1 and her parents.

Target cell binding at single cell level
The percentage of conjugate formation after the binding between a single lymphocyte and a K562 target cell was within the reference range for all the individuals tested (data not shown).

NK cell lytic activity of lymphocytes incubated with IFN-α and IFN-β
After incubation of lymphocytes with IFNs the NK cell activity showed a general increase in all the patients and their parents (Fig. 4). In the XP father group, the lytic activity increased up to the level observed in IFN-incubated lymphocytes of normal subjects. In contrast, the NK activity (measured after incubation with IFNs) in XP patients and their mothers reached the level observed in unstimulated cells from normal subjects, but was still significantly lower than that observed in normal

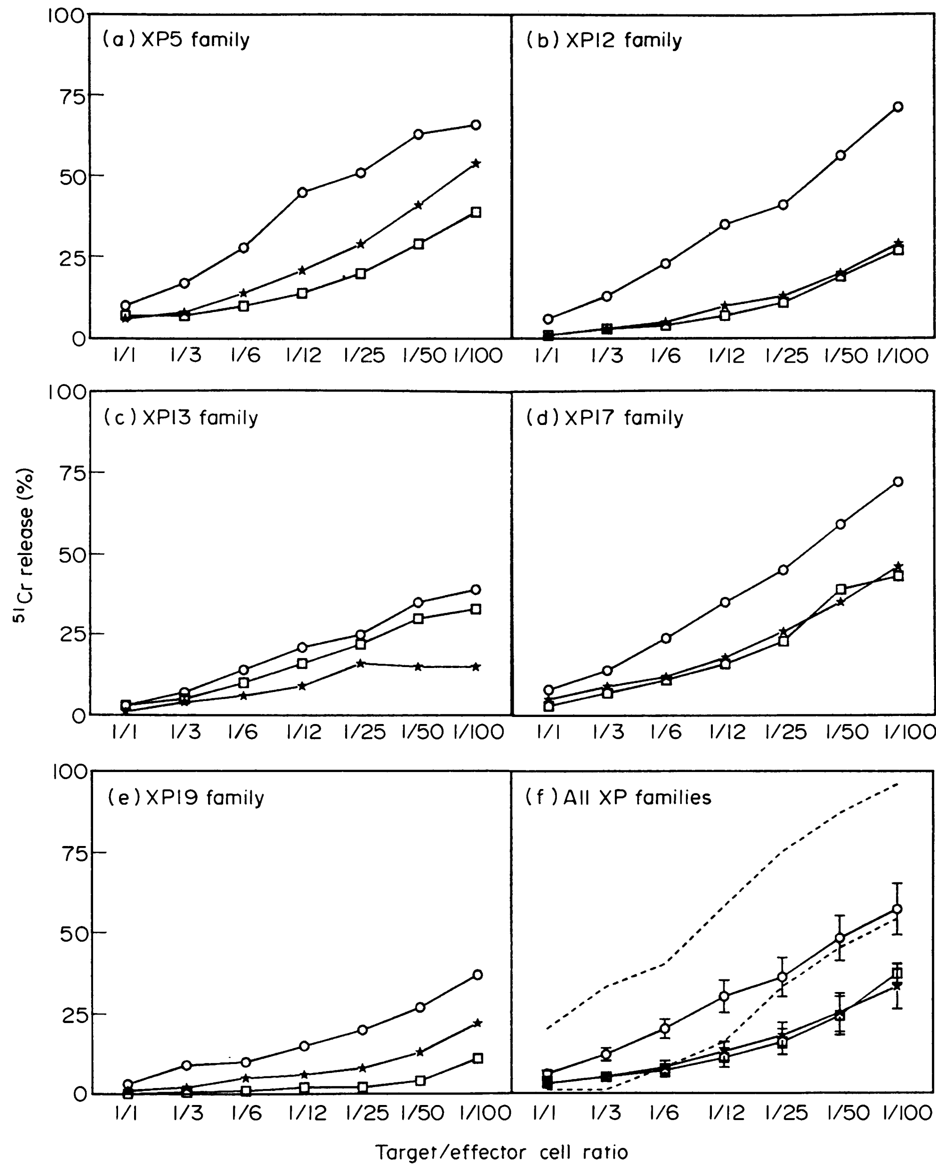

Fig. 2. Natural killer (NK) cell lytic activity in the members of XP families: ★, five children affected by XP; ○, five fathers; and □, five mothers (a, b, c, d, e). (f) The dashed lines represent respectively the upper and lower limits of the reference range of the response in 29 matched healthy subjects. Results are expressed as mean % ± s.e.m.

lymphocytes incubated with IFN-α ($P<0{\cdot}027$ and $P<0{\cdot}006$) or IFN-β ($P<0{\cdot}001$).

In the TTD families, only the lymphocytes of TTD fathers incubated with IFN-β reached the range of variability of the unstimulated normal lymphocytes, while the increased activity observed in all the other samples never reached normal values ($P<0{\cdot}01$ at least). The differences in NK cell activity after stimulation were most evident compared with the values of stimulated normal cells ($P<0{\cdot}003$ at least).

DISCUSSION

Recent observations have suggested that cancer proneness in XP patients is not exclusively related to persistent DNA lesions determined by a defective DNA repair after exposure to u.v. light, but could result from a more complex situation. Norris *et al.* [11] suggested that an abnormally low lytic activity found in NK cells from the peripheral blood of XP patients, together with the increased u.v. light-induced mutation, may contribute to the greatly enhanced susceptibility to skin cancer in XP patients.

This hypothesis seemed to be further supported by the observation that impaired NK activity was not seen in the patient affected by TTD [12]. Although the same genetic defect of XP group D is present in TTD patients, there is no evidence of the severe cutaneous alterations and skin tumours typical of XP. This may be attributed to the young age of TTD patients or to the fact that their skin is dry and ichthyotic, or to the involvement of unidentified factors that contribute differentially to the carcinogenic effect of u.v. light in XP and in TTD.

Based on these observations, full-blown clinical manifestations in XP appear to result from recessive mutations at two independent loci, one controlling DNA repair, the other controlling NK activity [19]. This hypothesis implies that a critical factor responsible for cancer proneness in XP patients

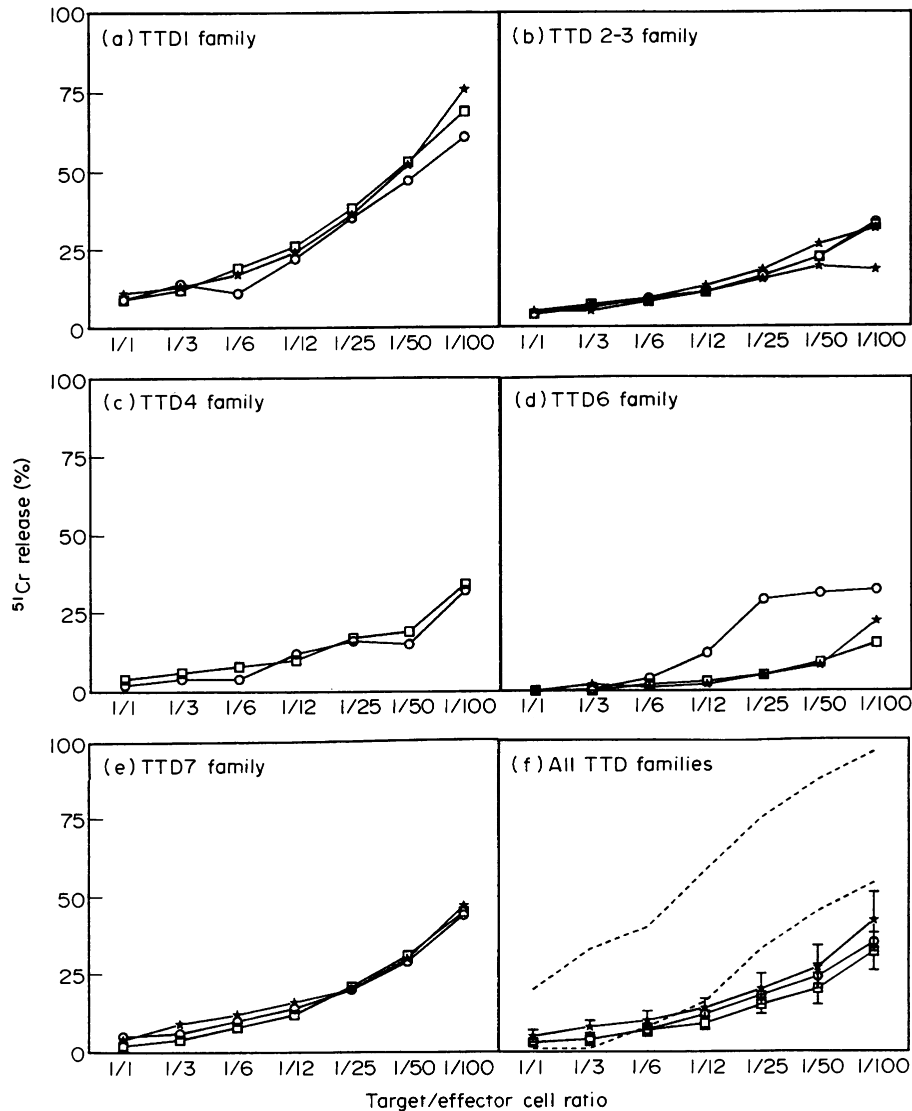

Fig. 3. Natural killer (NK) cell lytic activity in the members of TTD families: ★, five children affected by TTD; ○, five fathers; and □, five mothers (a, b, c, d, e). (f) The dashed lines represent respectively the upper and lower limits of the reference range of the response in 29 matched healthy subjects. Results are expressed as mean % ±s.e.m.

might be a congenital defect in NK activity, hampering the normal immune surveillance process for preventing tumour outgrowth [20].

In this study NK cell lytic activity was decidedly low in five XP patients (complementation groups C and D) and impaired in XP mothers but not in XP fathers.

The results among the TTD families varied: one family showed completely normal NK activity, in agreement with Norris *et al.* [12] while NK activity was decreased in the other four patients and their relatives, starting from grandparents.

These data indicate that reduced NK activity is not confined to XP patients. Further, it is probably not related to malignancies and/or differential exposure to sunlight or to an accumulation of DNA damage due to the impaired excision repair pathways, but is genetically determined, and it is independent of XP mutations.

The normal numbers of circulating $CD16^+$ cells and their normal cell binding to tumour targets observed in all individuals with decreased NK cell activity indicate a functional defect in the lytic machinery of NK cells.

In TTD family members, NK activity increases after incubation with IFN-α or IFN-β, but never reaches normal values. In XP patients and their mothers, the defect is almost completely corrected *in vitro* by IFN, suggesting that it may reside in the maturation of circulating NK cells or in the basal plasma level of the cytokines involved in these maturation steps. Recently, decreased production of IFN-γ, a potent inducer of NK activity, has been reported in XP leucocytes stimulated with poly inosinic cytidylic acid [21].

Previous studies reported that in XP patients $CD3^+CD4^+$ lymphocytes were reduced [22], but no alteration was found in the $CD4^+/CD8^+$ ratio [11,12,22]. Our data indicate that three out of five XP patients and their fathers had low numbers of circulating $CD3^+CD4^+$ cells with a normal proportion of $CD8^+$ and $CD16^+$ cells. The deficient number of $CD4^+$ helper cells is not present in XP mothers or in any members of the TTD

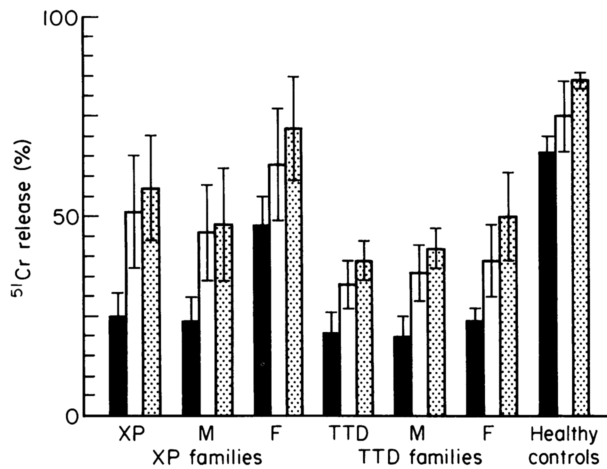

Fig. 4. Effect of interferon incubation on natural killer (NK) cell lytic activity of members of the five XP and five TTD families. Results are expressed as mean % ±s.e.m. for the target/effector cell ratio 1/50. ■, Unstimulated; □, IFN-α; ▦, IFN-β. M, mothers; F, fathers.

families. It seems unlikely that the decreased number of CD3^{+}CD4^{+} cytokine-producing T cells found in XP patients is responsible for a maturation defect of NK cells, since XP fathers have low CD4^{+} cells and normal NK lytic activity, and XP mothers and all TTD family members have normal numbers of helper cells and generally low NK cell lytic activity.

Since it is well known that T cells play a major role in the control of neoplasia, the association between a NK activity defect (present in XP and TTD patients) and the low number of CD3^{+}CD4^{+} lymphocytes (observed only in XP patients) may account for the increased risk of cancer in XP. This possibility is further supported by the fact that XP heterozygotes show either low NK activity but normal numbers of helper cells (XP mothers) or normal NK activity but low numbers of helper cells (XP fathers).

Our study indicates that T cell changes together with DNA repair defects and reduced NK activity could be involved in the increased cancer risk in XP patients.

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