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MEASUREMENT OF IN VIVO HGPRT-DEFICIENT MUTANT CELL FREQUENCY USING A MODIFIED METHOD FOR CLONING HUMAN PERIPHERAL BLOOD T-LYMPHOCYTES

ヒト末梢血Tリンパ球クローニング改良法を用いた生体内HGPRT欠損突然変異細胞頻度の測定

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リンパ球の分離を行われた渡辺芳子氏，単クローン抗体を用いてリンパ球コロニーの染色，解析に技術援助と忍耐を提供された尾崎恭子，岡光久恵の各氏に感謝の意を表したい．また，この原稿の検討に力添えくださったMichael A. Bean並びに中村 典両博士に深く感謝する．原稿をタイプしていただいた高木迪子，若狭光枝の両氏の協力に謝意を表したい．

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Mutation Research

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SUMMARY

Approximately 80% of human peripheral blood T-lymphocytes could be cloned in the presence of crude Interleukin-2, phytohemagglutinin, and X-irradiated autologous lymphocytes and Raji B-cells. This modified cloning method was used to measure the in vivo frequency of HGPRT-deficient mutant T-lymphocytes. Repeated experiments using blood from the same individuals revealed that the frequency of mutant cells was almost constant for each individual even though the cloning efficiency of lymphocytes varied somewhat from experiment to experiment.

Approximately 80% of both wild-type unselected and 6-thioguanine-resistant colonies had helper/inducer and about 20% had suppressor/cytotoxic T-lymphocyte markers. No difference was observed in the distribution of lymphocyte subsets between wild and mutant lymphocyte colonies.

INTRODUCTION

It is now well established that many carcinogens are mutagens.[1,2] Although the mechanism of induction of human cancers by carcinogens is not yet clear, it seems reasonable at present to assume that an individual's frequency of somatic cell mutants is one indicator of exposure to environmental carcinogens. In order to assess the in vivo mutation frequency in humans, the use of peripheral blood cells is the most convenient in terms of ready availability and large numbers. Of these blood cells, lymphocytes have long been used for chromosome studies.[3]

要 約

インターロイキン2 (IL2), フィトヘマグルチニン並びにX線照射自己リンパ球及びRaji B細胞を用いることにより, ヒト末梢血Tリンパ球の約80%をクローニングできた. この改良クローニング法を用いて, HGPRT欠損突然変異Tリンパ球の生体内頻度を測定した. 同一対象者の血液を用いて繰り返し実験を行ったところ, リンパ球のクローニング効率は実験によって多少異なるが, 突然変異細胞の頻度は各対象者についてほぼ一定した値が得られた.

野性型非選択コロニー及び6-チオグアニン耐性コロニーの約80%はhelper/inducer Tリンパ球のマーカーを, 約20%はsuppressor/cytotoxic Tリンパ球のマーカーを表現していた. 野性型リンパ球コロニーと突然変異リンパ球コロニーの間でリンパ球サブセットの分布に差異はみられなかった.

緒 言

多くの発癌性物質が突然変異原であることが確認されている.[1,2] 発癌性物質によるヒトの癌誘発のメカニズムは明らかにされていないが, 突然変異体細胞の頻度は, 個人の発癌性環境因子への曝露の一つの指標と考えても今のところ問題はないであろう. ヒトの生体内突然変異の研究に末梢血細胞を用いるのは, それが直ちに入手可能であり, また多量に得られるので最も都合のよい方法である. これらの血液細胞の中で, リンパ球は染色体研究に長い間用いられてきた.[3] 最近の試験管内細胞培養技術の

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The recent development of in vitro cell culture techniques, especially the discovery and utilization of Interleukin-2 (IL2),[4] has enabled investigators to use lymphocytes for mutation studies as well. These mutation studies have exclusively used the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus as a marker because it is an X-chromosome-located gene and only one X-chromosome is active in somatic cells irrespective of the sex of donor. Therefore, a single alteration of the gene is sufficient to result in a phenotypic change. HGPRT-deficient mutant cells can be readily recognized by their resistance to the purine analogue, 6-thioguanine (TG).

The measurement of the in vivo mutant cell frequency of peripheral blood lymphocytes was first attempted by means of autoradiography to detect cells which incorporated [^{3}H]thymidine in the presence of TG.[5-7] However, it became apparent that while most of the lymphocytes are in G_0 stage in vivo, a small fraction of them are cycling and some of these cells are scored as resistant to TG.[8] Hence, the estimated mutant cell frequency is erroneously high when using this method. In order to circumvent this problem, Albertini et al[9] developed a mutation assay using IL2-dependent T-lymphocyte cloning and TG selection. They found a mutant frequency of 1.2×10^{-5} for peripheral blood lymphocytes from two donors. They also demonstrated that the 6-thioguanine-resistant (TG^r) lymphocyte colonies lacked HGPRT activity, and the mutant phenotype was stable after further culture without TG. Morley et al[10] also studied HGPRT gene mutation frequency using a T-lymphocyte cloning method. However, they found a mean mutant frequency of 3.0×10^{-6} for 14 individuals which is about four times lower than the frequency reported by Albertini et al.[9] On the other hand, Vijayalaxmi and Evans[11] reported a mean TG^r lymphocyte frequency of 1.4×10^{-5} for 26 donors which is comparable with the data from Albertini et al.

There are a number of possible explanations for these reported differences in TG^r lymphocyte frequency, the most important of which are the differences in culture methods, i.e., the use and kind of feeder cells and IL2. The importance of culture conditions is highlighted by Albertini's[12] finding of a tendency for the TG^r lymphocyte frequency to be overestimated when the non-

進歩，とりわけIL2の発見並びに利用によって，[4] リンパ球が突然変異研究にも使用可能となった．こうした突然変異研究では，専らhypoxanthine-guanine phosphoribosyl transferase (HGPRT) locusがマーカーとして利用されてきたが，それはHGPRT locusがX染色体上に存在する遺伝子で，体細胞内で実際に働いているX染色体は，提供者の性別にかかわりなく1個しかないという理由からである．よって表現型の変化には，遺伝子が1個変異するだけで十分である．HGPRT欠損突然変異細胞は，プリン構造類似体である6-thioguanine (TG) に対して抵抗性を示すことから容易に確認される．

末梢血リンパ球の生体内突然変異細胞頻度測定は，最初，オートラジオグラフィーによってTG存在下で[^{3}H] thymidineを取り込む細胞を検出することにより試みられた．[5-7] ところが，リンパ球のほとんどは生体内でGo段階にあるが，一部の分裂周期に入っているリンパ球の中にはTG耐性として記録されるものがあることが明らかとなった．[8] そのために，この測定法を用いると推定突然変異細胞頻度が，見当違いに高くなる．Albertiniら[9] はこの問題を解決するために，IL2依存性Tリンパ球クローニングとTG選択を用いた突然変異測定法を開発し，2名の提供者からの末梢血リンパ球の突然変異細胞頻度，1.2×10^{-5}を得た．また彼らは，6TG耐性 (TG^r) リンパ球コロニーにはHGPRT活性がなく，突然変異体表現型は，TG非存在下に更に培養した後も，不変であることも示した．Morleyら[10] もTリンパ球クローニング法によるHGPRT突然変異細胞の検出を試みたが，検出された14人の対象者の平均突然変異細胞頻度は3.0×10^{-6}で，これはAlbertiniら[9] によって報告された頻度の約4分の1である．一方，Vijayalaxmi と Evans[11] は26人の提供者の平均TG^rリンパ球頻度1.4×10^{-5}を報告しているが，これはAlbertiniらのデータに近い値である．

これまでに述べたTG^rリンパ球頻度の違いには多くの理由が考えられるが，最も重要なのが使用するfeeder細胞とIL2の種類等の培養方法の違いである．Albertini[12] は，非突然変異リンパ球のクローニング効率 (CE) が低い場合，TG^rリンパ球頻度が過大に見積もられる傾向を発見しているが，このことは

mutant lymphocyte cloning efficiency (CE) was low. Thus, we modified the previously reported methods, including the method of producing IL2 and feeder cells, and tried to make the conditions of cloning similar for nonmutant cells and mutant cells. This modification resulted in an improved CE of nonmutant lymphocytes. Several aspects of the mutation assay using this modified T-lymphocyte cloning are presented here.

Materials and Methods

Cell Culture Media. For maintenance of Raji-cells, RPMI 1640 medium containing 8 mM HEPES (Gibco), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated-screened fetal calf serum (HI-FCS) was used. For T-lymphocyte cloning assays, MEM-ALPHA (Gibco) containing 8 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10% HI-FCS, and 0.7 μg/ml phytohemagglutinin-P (PHA-P, Difco) was supplemented with 20% conditioned medium as an exogenous source of crude human IL2, to give growth medium (GM).

Preparation of Peripheral blood mononuclear Cells (PBMC). Venous peripheral blood was collected and defibrinated with glass beads. PBMC were recovered by Ficoll-Hypaque density centrifugation and washed twice with Earl's balanced salt solution (EBSS, Nissui, Japan).

Preparation of Conditioned Medium as a Source of Human IL2. Operatively resected human spleen was cut into small pieces with scissors and pressed through a stainless steel grid (pore size:500 μm) to produce a cell suspension. The cells were washed with EBSS containing 20 units/ml heparin and resuspended at 10^6 cells/ml in Eagle's MEM containing 8 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 2.5×10^{-5} M 2-mercaptoethanol, 1% PHA-M (Difco), and 2.5% HI-FCS. The cells were incubated at 37° C in humidified 5% CO_2. The culture supernatant was harvested after 24 hours and the cells were resuspended with the same fresh medium. The cells were cultured for another 24 hours, and the supernatant was collected. The supernatants were mixed, concentrated, and dialyzed against Eagle's MEM using a hollow fiber dialyzer/concentrator (Amicon).

培養条件の重要性を示している．したがって，我々はIL2 の産生方法並びに feeder 細胞等，以前報告された方法に変更を加えることにより，非突然変異細胞と突然変異細胞のクローニング条件を同様にすることを試みた．その結果，非突然変異リンパ球の CE が高くなった．本報では，改良Tリンパ球クローニング法を使用した突然変異測定法の幾つかの側面について述べる．

材料及び方法

細胞培養液． Raji 細胞の維持には，RPMI1640 培養液に次のものを加えたものを用いた．8mM HEPES (Gibco)，100units/ml penicillin，100μg/ml streptomycin，2mM L-glutamine 並びに10%熱不活性ウシ胎児血清(HI-FCS)．Tリンパ球クローニングには，8mM HEPES，100units/ml penicillin，100μg/ml streptomycin，2mM L-glutamine，10% HI-FCS，並びに 0.7μg/ml phytohemagglutinin-P(PHA-P, Difco) を含む MEM-ALPHA(Gibco)に，ヒトIL2 源として20% conditioned medium を加え，増殖培養液(GM)を作成した．

末梢血単核細胞(PBMC)の分離． 末梢静脈血を採取して，ガラスビーズで脱フィブリンした．Ficoll-Hypaque 比重遠心法で PBMC を分画し，Earl's balanced salt solution (EBSS, Nissui, Japan) で2回洗浄した．

ヒトIL2 源としてのconditioned medium の作成． 手術で切除されたヒト脾臓をはさみで細切し，ステンレス鋼グリッド(孔直径：500μm)に通し，細胞浮遊液を作った．細胞を 20units/ml heparin を加えた EBSS で洗浄し，8mM HEPES，100units/ml penicillin，100μg/ml streptomycin，2mM L-glutamine，2.5×10^{-5} M 2-mercaptoethanol，1% PHA-M (Difco)，2.5% HI-FCS を加えた Eagle's MEM に 10^6個/ml の割合で再浮遊させ，37° C 5% CO_2 の条件で培養した．培養上清を24時間後に回収した後，細胞は同一の新鮮な培養液で再浮遊した．更に24時間細胞を培養し，上清を集めた．これらの上清は hollow fiber dialyzer/concentrator (Amicon) を使って混合，濃縮し，Eagle's MEM に対して透析した．

Feeder Cells. A human Burkitt's lymphoma cell line, Raji, was a gift from Dr. Bean (Seattle, WA). Raji cells were irradiated with 10,000 rad from a 40 kVp X-ray source. Autologous PBMC were irradiated with 5,000 rad from the same source.

T-lymphocyte Cloning Assay. T-lymphocyte cloning assays were run entirely in 96-well, flat-bottomed microtiter plates (Costar). PBMC with or without feeder cells were placed in the GM (200 μl/well) with the indicated TG concentration. Each well without TG (control plate) received one PBMC and each well with TG (selection plate) received 10^5 PBMC. The medium was partially replaced (100 μl) on days 3, 8, and 13. On day 15, each well was observed with an inverted microscope to determine the presence or absence of colonies. CE was calculated for each plate from the ratio of colony-negative wells, assuming a Poisson distribution of cells with the ability to form colonies in wells. Frequency of TG^r cells was obtained by dividing CE of the selection plate by CE of the control plate.

Thymidine and Hypoxanthine Incorporation Assay. For the measurement of thymidine incorporation, cells in each well were labeled on day 15 with 0.5 μCi of [^{3}H]thymidine (20 Ci/mmol, New England Nuclear). After 18 hours, the contents of each well were collected separately onto glass fiber filters using a multiple cell harvester, and the radioactivity was measured by scintillation spectrophotometry. For the hypoxanthine incorporation assay of HGPRT, each well was labeled on day 15 with both 0.5 μCi of [^{3}H]thymidine and 0.05 μCi of [^{14}C]hypoxanthine (49.0 mCi/mmol; New England Nuclear). After 18 hours, well contents were harvested as above and the radioactivity was measured.

Detection of Cell Surface Markers. On day 15, contents of colony-positive wells were transferred to 17 x 17 mm wells (Costar) and expanded. The cells were incubated with mouse monoclonal antibodies Leu 4, Leu 2a, and Leu 3a (Becton Dickinson); this was followed by incubation with goat antimouse fluorescein isothiocyanate-labeled antibodies (TAGO). Cells were scored by fluorescence microscopy; 100 cells were counted for the number of positive cells from each colony.

Feeder 細胞. ヒト Burkitt リンパ腫細胞株, Raji は Bean 博士(Seattle, WA)から提供された. Raji 細胞に, 40kVp X線源から 10,000rad を照射した. 自己 PBMC も同一の線源から 5,000rad を照射した.

Tリンパ球クローニング. Tリンパ球クローニングは, 常に96穴の平底 microtiter plates (Costar) を用いて行った. PBMC を feeder 細胞と共に又は feeder 細胞なしに表示された TG 濃度で GM (200 μl/well) に入れた. TG を含まない各 well (control plate) には1個の PBMC を, TG を含む各 well (selection plate) には 10^5 個の PBMC を入れた. 培地は培養3, 8, 13日目に部分的(100 μl)に交換した. 15日目には, コロニー有無の確認のため各 well を倒立顕微鏡で観察した. Well 内にコロニーを形成する能力をもつ細胞がポアソン分布をとると想定して, 各 plate ごとにコロニーが形成されていない well の割合から CE を求めた. TG^r 細胞の頻度については, selection plate の CE を control plate の CE で割って求めた.

Thymidine, Hypoxanthine 取り込み測定. Thymidine 取り込みを測定するために, 各 well 内の細胞を培養15日目に, 〔^{3}H〕thymidine (20Ci/mmol, New England Nuclear) 0.5 μCi を加えて標識した. 18時間後, 各 well 内の細胞は multiple cell harvester を使ってガラスファイバーフィルター上に別々に集め, 放射活性を scintillation 分光光度法で計測した. HGPRT の hypoxanthine 取り込みによる測定では, 各 well ともに 〔^{3}H〕thymidine 0.5 μCi, 〔^{14}C〕hypoxanthine 0.05 μCi (49.0mCi/mmol; New England Nuclear) を, 培養15日目に加え標識した. 18時間後に, 前述の方法で well 内の細胞を採取し, 放射活性を計測した.
放射活性を計測した.

Cell surface marker の検出. 培養15日目, colony を形成した well 内の細胞を 17×17mm well (Costar) に移し, 増殖させた. 細胞はマウス単クローン抗体 Leu 4, Leu 2a 及び Leu 3a (Becton Dickinson) と反応させ, 引き続き, ヤギ抗マウスフルオレセインイソチオシアネート標識抗体 (TAGO) を使って標識を行った. 蛍光顕微鏡法によって1コロニー当たり細胞100個中の陽性細胞数を測定した.

RESULTS

Effect of Feeder Cells on the CE of TG^r Lymphocytes

PBMC from two volunteers were used to evaluate the feeder effect of X ray-inactivated Raji cells. Figure 1 shows that when 10^5 PBMC were cultured in the presence of 2.5 μg/ml TG, the CE of TG^r cells increased from 0.21×10^{-5} to 0.39×10^{-5} in one case and from 0.053×10^{-5} to 0.27×10^{-5} in another when 10^4 Raji cells were added as feeders. Thus, addition of Raji cells increased the CE 2-5 times. [^{3}H]thymidine incorporation revealed that the lymphocyte colonies growing in the presence of Raji cells were more radioactive than those without them, demonstrating the addition of Raji cells not only increased the CE but also increased the size of each colony. Therefore, in subsequent experiments, X-irradiated Raji cells were used as feeder cells (10^4 cells/well).

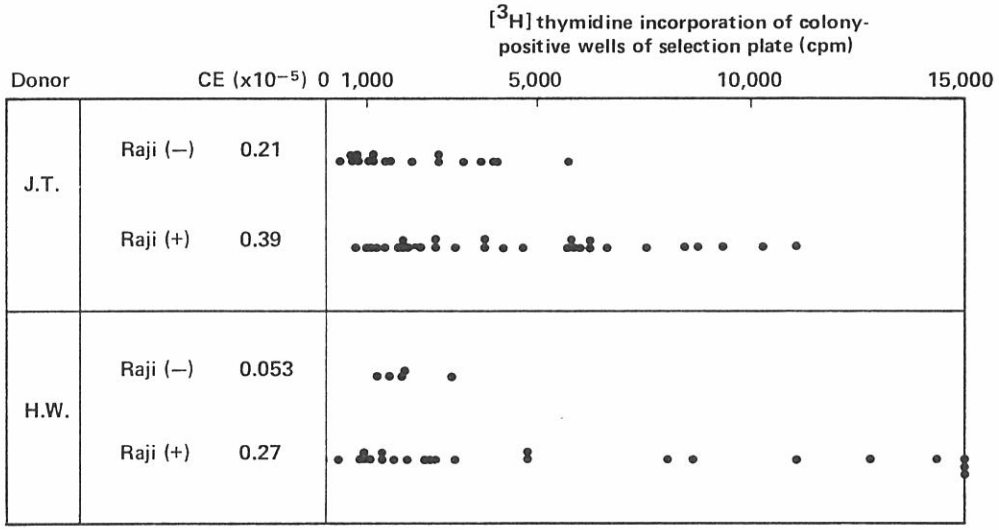
結　果

TG^r リンパ球の CE に及ぼす feeder 細胞の効果

2名の PBMC を用いて X 線照射した Raji 細胞の feeder 効果をみた．図1で示すように，10^5 個の PBMC を 2.5μg/ml TG の存在下で培養した場合，10^4 個の Raji 細胞を feeder として加えることにより，TG^r 細胞の CE は1例では 0.21×10^{-5} から 0.39×10^{-5} に，ほかの1例では 0.053×10^{-5} から 0.27×10^{-5} に増大した．このように，Raji 細胞の添加で CE は2～5倍に増大した．Raji 細胞存在下で増殖したリンパ球コロニーは，Raji 細胞を含まないコロニーに比べて〔^{3}H〕thymidine 取り込みが高いことがわかり，そのことから Raji 細胞の添加は，CE だけでなく各コロニーの大きさも増大させることが判明した．したがってその後の実験では，X 線照射した Raji 細胞を feeder 細胞（10^4 個/well）として用いた．

FIGURE 1 EFFECT OF IRRADIATED RAJI CELLS ON CLONING EFFICIENCY (CE) AND THYMIDINE INCORPORATION OF COLONY-POSITIVE WELLS OF SELECTION PLATE

図1　Selection plate のクローニング効率（CE）並びにコロニー形成 well の thymidine 取り込みに対する X 線照射 Raji 細胞の影響



Peripheral blood mononuclear cells (10^5 cells/well) with or without irradiated Raji cells (10^4 cells/well) were cultured in 96-well microtiter plates with growth medium containing 2.5 μg/ml of thioguanine (TG). On day 15, each well was observed with an inverted microscope to determine the presence or absence of lymphocyte colonies. CE was calculated from the proportion of colony-negative wells. Thymidine incorporation into colony-positive wells was measured by labeling cells with 0.5 μCi [^{3}H]thymidine for 18 hours after 15 days culture.

末梢血単核細胞（10^5個/well）を，X 線照射 Raji 細胞（10^4個/well）の存在下又は非存在下に thioguanine（TG）2.5μg/ml を含む増殖培養液を用いて96穴 microtiter plate で培養した．培養15日目に倒立顕微鏡で各 well を観察して，リンパ球コロニーの有無を決定した．CE はコロニーのない well の比率から求めた．コロニーが形成された well への thymidine 取り込みは，培養15日後に〔^{3}H〕thymidine 0.5μCi を用いて18時間，細胞を標識して測定した．

Determination of Thioguanine Concentration

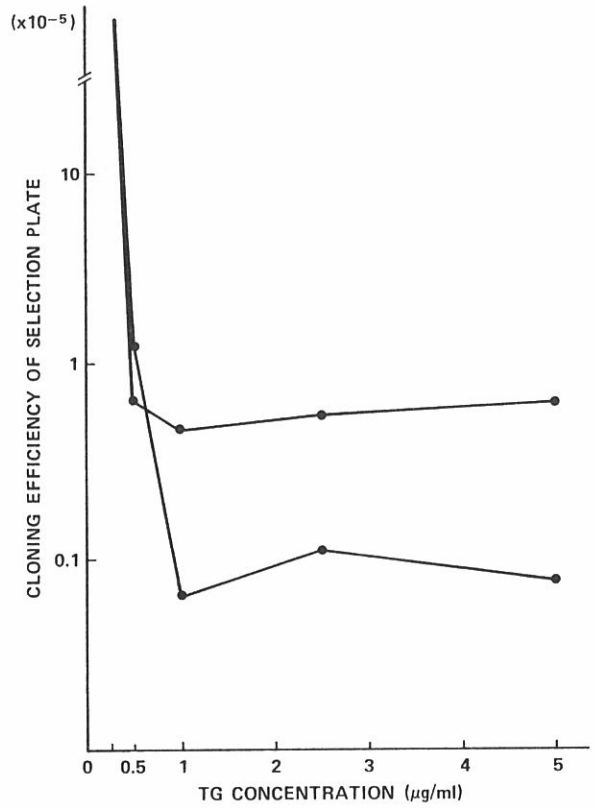
CE of TG^r cells was determined with different concentrations of the drug for two healthy donors. As shown in Figure 2, all the wells showed cell proliferation below 0.25 μg/ml of TG while the CE sharply decreased at TG concentrations of 0.25 to 1 μg/ml and remained constant between 1 to 5 μg/ml. Therefore, 2.5 μg/ml of TG was adopted in the following experiments.

Thioguanine 濃度の決定

TG^r 細胞の CE を，2名の健康な提供者について異なる濃度の薬剤で決定した．図2で示すように，TG 0.25μg/ml 以下ではすべての well で細胞増殖が見られたが，TG 濃度が 0.25μg/ml から 1μg/ml の間では CE は急激に減少し，1μg/ml から 5μg/ml の間で一定していた．したがって TG 2.5μg/ml を以下の実験に用いた．

FIGURE 2 DEPENDENCE OF THE CLONING EFFICIENCY (CE) OF SELECTION PLATE ON THIOGUANINE (TG) CONCENTRATION

図2 Selection plate のクローニング効率（CE）の thioguanine（TG）濃度への依存



Peripheral blood mononuclear cells (10^5 cells/well) and irradiated Raji cells (10^4 cells/well) were cultured in 96-well microtiter plates with growth medium containing the indicated dose of TG for 15 days. The presence or absence of lymphocyte colonies was determined by the observation with an inverted microscope and the CE was calculated from the proportion of colony-negative wells.

末梢血単核細胞（10^5個/well）と X 線照射 Raji 細胞（10^4個/well）を，図中に示す TG 量を含む増殖培養液を用いて15日間，96穴 microtiter plate で培養した．リンパ球コロニーの有無は倒立顕微鏡で観察して決定し，CE はコロニーがない well の比率から求めた．

HGPRT Activity of TG^r Colonies

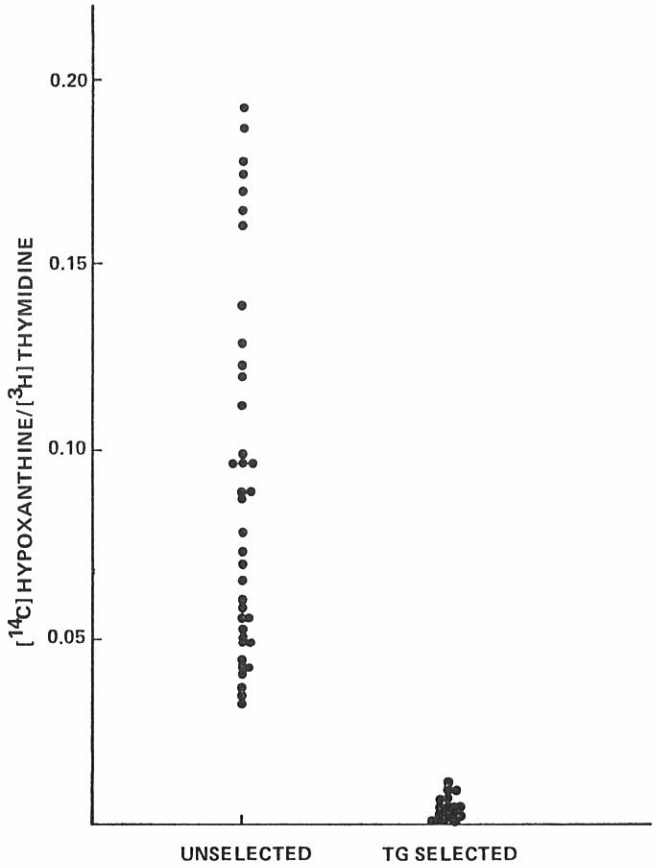
HGPRT activity of TG^r colonies was determined by labeling cells with both [^{3}H]thymidine and [^{14}C]hypoxanthine. Results are presented as the ratio of hypoxanthine incorporation to thymidine incorporation (Figure 3) to enable comparison of the results from colonies of different sizes. As shown in Figure 3, the ratios for the lymphocyte colonies in the TG plate were near zero, while the ratios for those colonies in the TG-free plate (unselected colonies) were much higher with an average of about 0.10. These results are consistent with the deficiency in HGPRT activity in the colonies.

TG^r コロニーの HGPRT 活性

TG^r コロニーの HGPRT 活性は，〔^{3}H〕thymidine，〔^{14}C〕hypoxanthine の両方で細胞を標識し，評価した．結果は，大きさの違うコロニーから得た結果が比較できるように hypoxanthine 取り込みと thymidine 取り込みとの比で示してある（図３）．図３に示すように，TG plate 内のリンパ球コロニーでは比がほぼ０であるのに対して，TG を含まない plate 内のリンパ球コロニー（非選択コロニー）での比は，平均約0.10と高かった．この結果は，コロニーの HGPRT 活性欠損と一致した．

FIGURE 3 HYPOXANTHINE INCORPORATION OF UNSELECTED AND THIOGUANINE (TG)-SELECTED COLONIES

図３ Unselected 及び thioguanine（TG）-selected コロニーからの hypoxanthine 取り込み



Cells cultured in wells of 96-well microtiter plates were labeled on day 15 for 18 hours with a mixture of 0.5 μCi of [^{3}H]thymidine and 0.05 μCi of [^{14}C]hypoxanthine. Selected and unselected colonies were isolated from the same individual.

96穴 microtiter plate の well で培養した細胞を，〔^{3}H〕thymidine 0.5μCi と〔^{14}C〕hypoxanthine 0.05μCi 両方で培養15日目に，18時間標識した．同一個人から，selected 及び unselected コロニーを得た．

Culture Conditions for the Control Plate

It is necessary for the accurate measurement of the TG^r cell frequency to achieve similar culture conditions for both selection and control plates. TG sensitive (TG^S) cells in the wells of the selection plate must have some effect on TG^r cells, even though the TG^S cells are only alive for the first four or five days of the culture with TG. In order to mimic more closely the culture conditions of the selection plate, 10^5 cells/well of autologous PBMC were X-irradiated and apportioned to the wells of the control plate in addition to 10^4 cells/well of irradiated Raji cells. As shown in Figure 4, the CE of the control plate increased from 0.25 to 0.37 in one person (1.5 times increase) and from 0.16 to 0.57 in the other (3.5 times increase) when the autologous PBMC were added. Also, in order to assess colony size in the presence or absence of the autologous PBMC, [^{3}H]thymidine incorporation by the colonies was measured. As shown in Figure 4, the X ray-inactivated autologous PBMC not only increased the CE but also supported clonal growth of the cells. Table 1 summarizes the culture conditions that we have used to measure TG^r mutant cell frequency in the following experiments.

Reproducibility of the Assay System

Since the modified lymphocyte cloning assay system was developed to study individual's TG^r cell frequency, demonstrable reproducibility of the system is very important. Two healthy donors were repeatedly examined over two years. The results are shown in Table 2. The first sample is designated time 0 and the time of subsequent samples listed as months after the first samples. In both cases, the mutant cell frequency was of the order of 10^{-6} and the TG^r cell frequency for each individual was almost constant over time (maximum difference was 50% in donor S.K.) although the CE of the control and selection plates varied somewhat from experiment to experiment. Also, donor J.T. always exhibited a higher mutant cell frequency than donor S.K.

Control plate の培養条件

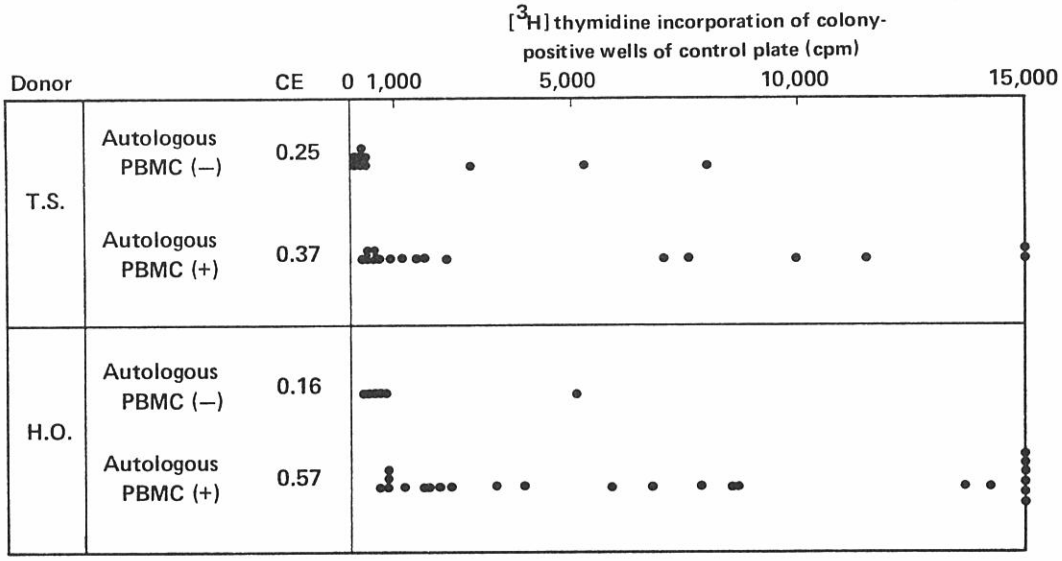
TG^r 細胞頻度を正確に測定するには，selection, control 両 plate での培養条件を同様のものにしなければならない．Selection plate の well 内の TG 感受性（TG^S）細胞は，TG で培養後 4 ～ 5 日しか生きてはいないが，TG^r 細胞に何らかの効果を与えるはずである．Selection plate の培養条件にもっと近付けるために，X線照射を受けた Raji 細胞 10^4 個/well のほかに，自己 PBMC 10^5 個/well を X線照射し control plate の well に分注した．図4に示すように，自己 PBMC を加えると，1 名の control plate の CE は0.25から0.37に増大し（1.5倍増），もう 1 名は0.16から0.57へと増大（3.5倍増）した．また，自己 PBMC の存在又は非存在下で，コロニーの大きさを査定するために，コロニーによる〔^{3}H〕thymidine 取り込みを測定した．図4に示すように，X線照射自己 PBMC は CE を増大させるだけでなく，細胞の増殖を助ける働きもした．表1は，以下の実験で TG^r 突然変異細胞頻度を測定するのに用いた培養条件の要約である．

測定法の再現性

この改良リンパ球クローニング法を，個人の TG^r 細胞頻度の研究に用いる際に，測定の再現性を示しておくことは重要である．2 名の健康な提供者を 2 年間繰り返し検査し，その結果を表2に示した．初回の標本は時間 0 とし，2 回目以降からの時間を初回後経過月数として示した．両名とも，突然変異細胞頻度は 10^{-6} のオーダーで，control, selection の両 plate の CE は実験によって多少変動したが，TG^r 細胞頻度はほぼ一定であった（S.K. の場合の最大差は50%であった）．また，J.T. は S.K. より高い突然変異細胞頻度を常に示していた．

FIGURE 4 EFFECT OF ADDITION OF IRRADIATED AUTOLOGOUS PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) ON THE CLONING EFFICIENCY (CE) AND THE THYMIDINE INCORPORATION OF COLONY-POSITIVE WELLS OF CONTROL PLATE

図4 Control plate におけるクローニング効率(CE)及びコロニー形成 well の thymidine 取り込みに対するX線照射自己末梢血単核細胞(PBMC)の影響



PBMC (1 cell/well) and irradiated Raji cells (10^4 cells/well) with or without irradiated autologous PBMC (10^5 cells/well) were cultured in 96-well microtiter plates with growth medium containing no TG. On day 15, each well was observed with an inverted microscope and the CE was calculated from the proportion of colony-negative wells. Thymidine incorporation into colony-positive wells was measured by labeling cells with 0.5 μCi of [^{3}H] thymidine for 18 hours after 15 days of culture.

PBMC(1個/well)及び照射Raji細胞(10^4個/well)を，照射自己PBMC(10^5個/well)の存在下又は非存在下にTGを含まない増殖培養液を用いて96穴 microtiter plate で培養した．培養15日目，倒立顕微鏡で各 well を観察し，コロニーが形成されていない well の比率から CE を求めた．コロニーが形成された well への thymidine 取り込みは，培養15日目に〔^{3}H〕thymidine 0.5μCi を用いて18時間，細胞を標識して測定した．

TABLE 1 CULTURE CONDITIONS FOR THE MEASUREMENT OF THE TG^r CELL FREQUENCY

表1 TG^r 細胞頻度測定の培養条件

		Control Plate	Selection Plate
Fresh PBMC[a] (cells/well)		1	10^5
Feeder cells			
Raji[b]	(cells/well)	10^4	10^4
Autologous PBMC[c]	(cells/well)	10^5	0
TG[d]	(μg/ml)	0	2.5

a: Peripheral blood mononuclear cells 末梢血単核細胞

b: Irradiated with 10,000 rad 10,000rad 照射

c: Irradiated with 5,000 rad 5,000rad 照射

d: 6-thioguanine 6-thioguanine

TABLE 2 REPRODUCIBILITY OF THE MEASUREMENT OF TG^r CELL FREQUENCY
表 2 TG^r 細胞頻度測定の再現性

Donor	Time (Months)	Control Plate			Selection Plate			TG^r cell Frequency $\times 10^{-6}$
		Total Wells	Positive Wells	CE[a]	Total Wells	Positive Wells	CE $\times 10^{-5}$	
J.T.	0	96	43	0.59	96	27	0.33	5.6
	3	96	39	0.52	96	22	0.26	5.0
	10	96	40	0.54	96	19	0.22	4.1
	22	96	27	0.33	96	16	0.18	5.5
S.K.	0	96	40	0.54	96	10	0.11	2.0
S.K.	12	96	32	0.41	96	10	0.11	2.7
	16	87	27	0.37	96	6	0.065	1.8
	24	89	27	0.34	96	6	0.065	1.9

[a]: Cloning efficiency クローニング効率

Surface Markers of the Mutant and Nonmutant Lymphocyte Colonies

A total of 119 colonies isolated either from control plates or from selection plates were examined for their lymphocyte surface markers (Table 3). All the colonies bore the pan-T-lymphocyte marker, Leu 4, indicating that they were derived from T-lymphocytes. The fraction of colonies having the Leu 3a(+) and Leu 2a(−) phenotype (characteristics of helper/inducer T-lymphocytes) was 47 out of 61 (77%) and 44 out of 58 (76%) for unselected and TG^r colonies, respectively. Thus, almost 80% of the colonies in both plates had the helper/inducer T-lymphocyte phenotype. The fraction of colonies showing suppressor/cytotoxic T-lymphocytes phenotype [i.e., Leu 3a(−) and Leu 2a(+)] was 10/61 (16%) for unselected colonies and 13/58 (22%) for TG^r colonies, a difference which is not statistically significant. The remaining five colonies had both markers, Leu 3a(+) and Leu 2a(+), and were the result of innoculation of more than one cell of different phenotype per well because the examination by the immunofluorescence double staining method revealed that individual cells were either Leu 3a(+) and Leu 2a(−) or Leu 3a(−) and Leu 2a(+).

突然変異及び非突然変異リンパ球コロニーの表面マーカー

Control, selection 両 plate から得た計119個のコロニーについてリンパ球表面マーカーを検索した(表3). 全コロニーが, T リンパ球に由来することを示す pan-T リンパ球マーカーの Leu 4 をもっていた. Leu 3a(+), Leu 2a(−) 表現型 (helper/inducer T リンパ球) をもつコロニーの割合は, 非選択コロニーが61コロニーのうち47(77%), TG^r コロニーが58コロニーのうち44(76%)であった. このように, 両 plate のコロニーの約80%が helper/inducer T リンパ球表現型を有していた. Suppressor/cytotoxic T リンパ球表現型〔すなわち Leu 3a(−), Leu 2a(+)〕を有するコロニーの割合は, 非選択コロニーが61コロニーのうち10(16%)及び TG^r コロニーが58コロニーのうち13(22%)であったが, 差異は統計的に有意でない. 残る5コロニーは Leu 3a(+), Leu 2a(+) 両方のマーカーをもっており, 免疫蛍光二重染色法で調べた結果, 個々の細胞が Leu 3a(+), Leu 2a(−) あるいは Leu 3a(−), Leu 2a(+) であったことから, それらのコロニーは, 1 well に異なった表現型をもつ2個以上の細胞が分注された結果と考えられた.

TABLE 3 SURFACE MARKERS OF COLONIES
表 3 コロニーの表面マーカー

	No. of Colonies	Leu 3a	Leu 2a	Leu 3a + Leu 2a
Unselected	61	47	10	4
TG selected	58	44	13	1

Correlations Between CE and Lymphocyte Subsets of PBMC

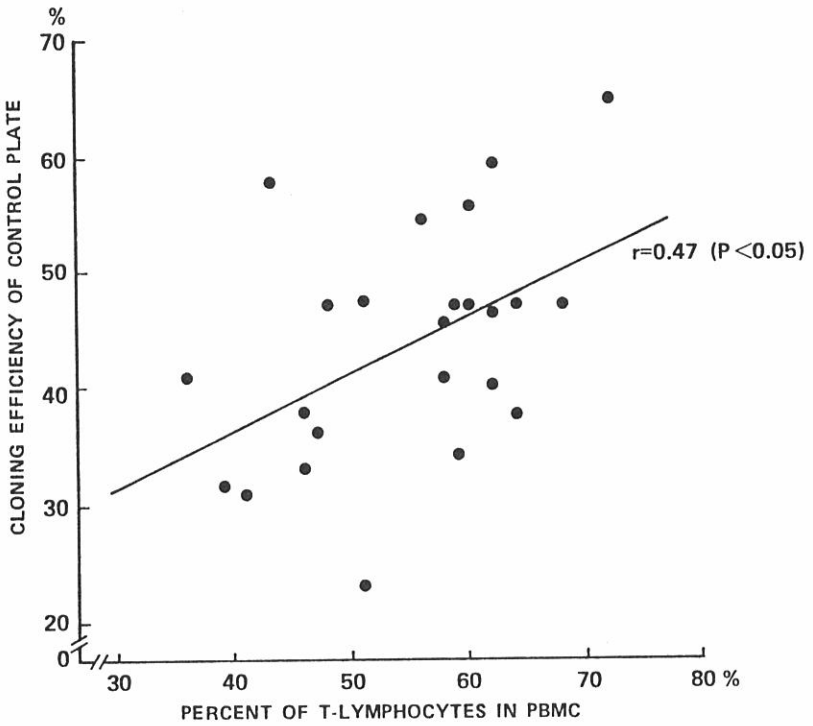
To assess the CE of lymphocytes of different subsets, the results obtained from control plates were compared to the PBMC subsets for 24 individuals. The CE of the control plates ranged from 23% to 65% (mean±1SD:44%±10%) and the proportion of T-lymphocytes [as defined by Leu 2a(+) or Leu 3a(+) cells] in the PBMC ranged from 36% to 72% (mean±1SD:55%±9.5%). The CE positively correlated with the proportion of T-lymphocytes (Figure 5). The

PBMC のリンパ球サブセットと CE の相関

各々のサブセットのリンパ球の CE を査定するために，24人について control plate の CE を PBMC サブセットと比較した．Control plate の CE は23%から65%に分布し（平均±1SD: 44%±10%），PBMC 中のTリンパ球〔Leu 2a（＋）又は Leu 3a（＋）細胞〕の割合は36%から72%（平均±1SD: 55%±9.5%）であった．CE はTリンパ球の割合と正の相関を示した（図5）．個人の CE と PBMC 中のTリンパ球の

FIGURE 5 CORRELATION BETWEEN THE PROPORTION OF T-LYMPHOCYTES (i.e., Leu 2a(+) or Leu 3a(+) Cells) IN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) AND THE CLONING EFFICIENCY (CE) OF CONTROL PLATE

図5 末梢血単核細胞（PBMC）のTリンパ球（Leu 2a（＋）あるいは Leu 3a（＋）細胞）の比率と control plate のクローニング効率（CE）の相関



A part of the PBMC which were used for clonal assay were stained with anti-Leu 2a or anti-Leu 3a monoclonal antibody and goat antimouse fluorescein isothiocyanate-labeled antibody. For each sample 100 cells were counted. PBMC from 24 donors were examined.

クローニングに用いた PBMC の一部を抗 Leu 2a 又は抗 Leu 3a 単クローン抗体，並びにヤギ抗マウスフルオレセインイソチオシアネート標本抗体を使って染色した．各標本について100個の細胞を観察した．提供者24人の PBMC について検索した．

ratio of the individual's CE to the proportion of T-lymphocytes in PBMC ranged from 0.39 to 1.0 with a mean of 0.78. This means that, on average, 78% of the T-lymphocytes [i.e., Leu 2a(+) plus Leu 3a(+)] in PBMC were cloned under these experimental conditions when considering all the colonies had T-lymphocyte surface phenotypes. The CE of control plates also positively correlated ($p<0.05$) with the proportion of Leu 3a(+) cells in the PBMC. As 80% of colonies from control plates were Leu 3a(+), CE×0.8 equals the percentage of clonogenic Leu 3a(+) cells in PBMC. The proportion of Leu 3a(+) cells in PBMC varied from 21% to 55% (mean±1SD:38%±9.2%). The ratio of the CE×0.8 to the proportion of Leu 3a(+) cells in PBMC varied from 0.53 to 1.0, with a mean of 0.88. This means that, on average, 88% of the Leu 3a(+) (helper/inducer) T-lymphocytes in PBMC were cloned in this assay system.

DISCUSSION

As the mutant frequency in this assay is derived by dividing the CE of the lymphocytes in the selection plate by the CE of the cells in the control plate, it was attempted to maintain similar culture conditions for the control and the selection plates. To this end, irradiated autologous PBMC were added, in addition to Raji cells, to the control plate as feeder cells to mimic conditions in the selection plate. The lack of difference in terms of T-cell surface markers between the classes of colonies from the control and selection plates might imply that good homology of culture conditions was achieved. However, the irradiated PBMC added to the control plate do not change the morphology in response to PHA in contrast to the TG^S cells in the selection plate. The TG^S cells in the selection plate exhibit blastic change with PHA stimulation and may produce or consume various lymphokines, including IL2, early in their culture. Thus, the addition of irradiated PBMC to the control plate is not precisely analogous to the presence of the TG^S cells in the selection plate. However, it is currently impossible to use feeder cells which behave the same as TG^S cells in the selection plate.

The CEs in the control plates increased 1.5-3.5 times due to the addition of autologous PBMC to supplement the Raji cells as feeder cells. This may be one of the reasons why we obtained a

割合との比率は，0.39から1.0にわたり平均値は0.78であった．このことは，全コロニーがTリンパ球表面表現型を有すると想定すると，平均してPBMC中のTリンパ球〔Leu 2a(＋)と Leu 3a(＋)〕の78%が，上記のような実験条件のもとでクローン化されたことを示す．また，control plate の CEは PBMC中の Leu 3a(＋)細胞の割合とも正の相関($p<0.05$)を示した．Control plate の80%のコロニーがLeu 3a(＋)であったので，CE×0.8 と PBMC 中のクローン形成能をもった Leu 3a(＋)細胞のパーセントが等しくなる．PBMC 中の Leu 3a(＋)細胞の割合は21%から55%(平均±1SD: 38%±9.2%)まで変化し，それに対する CE×0.8 の比率は0.53から1.0と変化し，その平均値は0.88であった．このことは，平均して PBMC 中の Leu 3a(＋)(helper/inducer)Tリンパ球の88%がこの方法でクローン化されたことを意味する．

考　察

この測定方法では，突然変異細胞頻度は selection plate のリンパ球 CE を control plate の CE で割って算出するので，control, selection 両 plate 内の培養条件をできるだけ近付けるように試みた．そのために，Raji 細胞のほかにX線照射自己 PBMC を feeder 細胞として control plate に加え，selection plate 内の培養条件に類似するようにした．control, selection 両 plate で得られたコロニーのT細胞表面マーカーに差異が見られなかったことは，培養条件が十分均質であったことを示しているのかもしれない．しかし，control plate に加えたX線照射 PBMC は，selection plate の TG^S 細胞とは違って PHA に反応せず形態を変えない．Selection plate の TG^S 細胞は PHA 刺激に反応して幼若化し，培養初期においては IL2 を含む種々のリンフォカインを産生したり，消費したりするであろう．このようにX線照射した PBMC の control plate への添加が，正確に selection plate 内の TG^S 細胞の存在と全く同質なものにはならない．Selection plate 内の TG^S 細胞と同じ挙動をする feeder 細胞を作製することは，現在，不可能である．

自己 PBMC を，feeder 細胞として Raji 細胞に添加した結果，control plate 内の CE が1.5から3.5倍増加した．このことは，恐らく我々が得た TG^r 細胞

lower TG^r cell frequency than that originally reported by Albertini et al[9] or Vijayalaxmi and Evans.[11] They used only irradiated human lymphoblastoid cell lines as feeder cells for the control plate, and their CE was somewhat lower than ours. According to a more recent report from Albertini,[12] TG^r cell frequency was of the order of 10^{-6}, being comparable to our results, with normal adult donors whose CE in the control plates was more than 30%. Also, he observed that TG^r cell frequency 'inversely' correlated with the corresponding CEs of the control plate. This inverse correlation was due mainly to assays in which the CEs in the control plates were less than 10%. Thus, it would appear that a culture system in which a high (true) CE in the control plates is obtained is necessary for an accurate mutation assay using the T-lymphocyte cloning method. In our system, the CE of control plates was about 40% (the maximum CE of a control plate is theoretically 40%-70% which is the percentage of T-lymphocytes in PBMC), and the reproducibility of the measurement of TG^r cell frequency was good.

In the method reported by Morley et al,[10] the CE in the control plate was 20%-60% and the mean mutant frequency obtained for normal adults was 3.0×10^{-6}. These results for both the CE in control plates and the mutant frequency are comparable to our results. But, there are several differences in the culture methods. Initially, they used only irradiated autologous lymphocytes as feeder cells in the control plates, and added no feeder cells into the selection plates. According to a more recent paper,[13] they changed their method and started to use both lymphocytes and a lymphoblastoid cell line as feeder cells for the control plate. In their method, they use a smaller number ($1\text{-}2 \times 10^4$) of lymphocytes than we and others (10^5 cells/well) in the wells of the selection plate because they use round bottom plates. Therefore, 5-10 selection plates must be set up to test a sufficient number of lymphocytes as in our method or in the method of other groups, which is more time consuming and uses a considerable amount of medium containing IL2.

The reason for the dominance of the helper/inducer T-lymphocyte subset phenotype on the colonies we obtained is probably because that helper/inducer lymphocytes constitute a larger subset of blood T-lymphocytes than do the

頻度が，最初に Albertini ら[9] や Vijayalaxmi と Evans[11] によって報告されたものより低かった原因の一つと考えられよう．彼らは control plate に feeder 細胞として照射したヒトB細胞株しか用いず，CE は幾分か我々の値より低かった．Albertini[12] の最近の報告によれば，control plate の CE が30％以上の健常な成人提供者の場合，TG^r 細胞頻度が 10^{-6} のオーダーで我々の得た値に近かった．彼はまた，TG^r 細胞頻度が対応する control plate の CE と負の相関を示すことも見いだした．この負の相関は，主に control plate の CE が10％未満の測定例の存在によるものであった．このようにTリンパ球クローニングによる突然変異測定法においては，control plate で高い（真の）CE が得られることが正確な値を得るために必要であろう．我々の方法では，control plate の CE は約40％（control plate の最大 CE は，理論的に PBMC 中のTリンパ球の割合に等しくなり40％から70％となる）で，TG^r 細胞頻度測定の再現性は良好であった．

Morley ら[10] が報告した方法では，control plate の CE は20％から60％で，健常な成人の平均突然変異細胞頻度は 3.0×10^{-6} であった．これら control plate の CE と突然変異細胞頻度は両方とも我々の得た結果に近いが，培養方法には多少の差異がある．最初に Morley らは，control plate に feeder 細胞としてX線照射自己リンパ球のみを使い，selection plate には feeder 細胞を添加しなかった．最近の論文[13] によれば，彼らは培養方法に変更を加え，control plate の feeder 細胞にリンパ球とB細胞株の両方を使用し始めた．彼らは丸底 plate を用いているため，selection plate の 1 well に分注するリンパ球数が我々や他の研究者が用いている数（10^5 個/well）より少ない（$1 \sim 2 \times 10^4$）．故に，我々あるいは他の研究者が用いる方法と同じだけの数のリンパ球を調べるためには，5から10枚の selection plate を用意しなければならず，時間がかかり，IL2 を含む培地をかなり使うことになる．

我々が得たコロニーで helper/inducer T リンパ球サブセット表現型が優性である理由は，suppressor/cytotoxic 細胞に比べて，helper/inducer リンパ球の方が末梢血中でより大きなTリンパ球サブセットを構成し，

suppressor/cytotoxic cells, and that PHA preferentially stimulates this subset.[14] If all the subsets could be stimulated equally, the CEs of both control and selection plates would be higher, resulting in a more precise measurement of the mutant frequency. Recently, Weber et al[15] reported that all the T-lymphocytes in human peripheral blood could be cloned by the use of an anti-CD3 monoclonal antibody and IL2. The CD3 molecule is present on the surface of all mature human T-lymphocytes[16] and is closely linked to the T-lymphocyte antigen receptor.[17] If this antibody triggering of T-lymphocytes could be adapted for the mutation assay in place of PHA, the measurement of TG^r lymphocyte frequency should become more precise.

Although some features still remain to be improved, we believe the current modified method for cloning T-lymphocytes is applicable to human-specific-locus mutagenicity monitoring. A study is now in progress to detect radiation-induced mutations in atomic bomb survivors in Hiroshima whose relative risk of cancer is reported to be higher than in the normal population.[18]

また PHA がこのサブセットを好んで刺激するためであろう.[14] 全サブセットが同じように刺激されれば, control 及び selection plate の CE はより高い値を示し, より精確な突然変異細胞頻度の測定ができるであろう. 最近, Weber ら[15] は, ヒト末梢血のTリンパ球はすべて, 抗 CD3 単クローン抗体と IL2 を用いてクローン化できることを報告した. CD3 分子はすべての成熟したヒトTリンパ球の細胞表面に存在し,[16] Tリンパ球抗原受容体と複合体を形成している.[17] この抗体によるTリンパ球刺激を PHA の代わりに突然変異測定に適用できれば, TG^r リンパ球頻度の測定はもっと正確になるはずである.

まだ改善の余地は残されているものの, 現在の改良を加えたTリンパ球クローニング法は, ヒトにおける特定遺伝子突然変異のモニタリングに適用可能である. 現在, 癌相対危険度が健常者より高いとされている広島の原爆被爆者において,[18] 放射線誘発による体細胞突然変異を調査する研究が進行中である.

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