1．健康食品の酸産生性の評価方法に関する検討

1－A．研究目的

食品摂取に伴うプラーク細菌の糖代謝による酸産生が、直接歯表面を脱灰し「う蝕」を発生させる原因となることから、健康食品全般においても酸の原因とならないこと、すなわち「う蝕」の原因とならないことが望まれる。過去3回の国際会議（米国 San Antonio：1986年、仙台：1994年、英国 London：1999年）のいずれでも、食品のう蝕誘発性能（＝酸産生性）を評価する方法の一つとして、食品摂取後のヒト・プラーク pH を測定することが「科学的に正しい方法」として推奨されている。そこで、現在本研究室にて行われている「食品の酸産生性検定システム」を詳細に検討することで問題点を抽出し、健康食品の有効性評価における酸産生性評価の位置付けについて考察した。

1－B．研究方法

1）現在、厚生労働省特定保健用食品認定を目的に、東北大学大学院歯学研究科口腔生物学講座口腔生化学分野にて行われている食品酸産生性の検定システムを、1)-1 信頼性、1)-2 実用性の2面から検討し、2）将来における酸産生性検定の位置付けについて考察した。

1－C．研究結果
1－D．考　　察

1）　酸産生性検定システムについて

1)-1 信頼性について

現在、東北大学大学院歯学研究科口腔生物学講座口腔生化学分野にて行われている食品酸産生性の検定システムには、電極内臓法（プラーク pH テレメトリー法）（図 1-1-1、1-1-2、1-1-3）が用いられている。

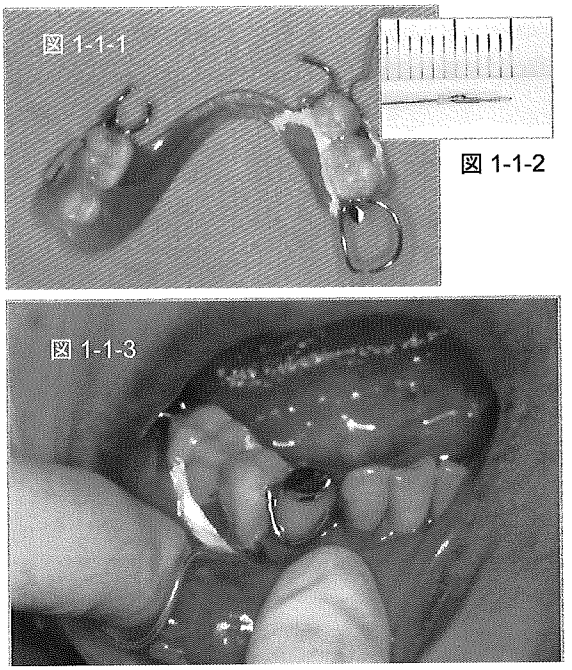

図 1-1　pH 電極内臓法の実際：
pH 微小トランジスター電極（図 1-1-1）を義歯へ埋め込み（図 1-1-2）、それを被験者の口腔内に設置しプラークを蓄積させ（図 1-1-3）、食品摂取時の pH 変化をモニターする

食品の酸産生性は食品摂取後のプラーク中の酸産生性（具体的には pH 低下程度）によって測定されるが、この方法は、口腔内に設置した pH 微小電極上にプラークを形成させ、口腔内で食品の酸産生性を測定するものであり、現段階では最も科学的信頼性が高い。実際、「う蝕の原因になりにくい食品」を検定するための酸産生性測定法として、国際トゥースフレンドリー協会（スイス）が 1982 年より採用しているものであり、長年にわたる酸産生性検定の実績をもつ。この方法は、現在に至るまでヨーロ

ッパとアジアを中心として*、う蝕予防用食品検定法のスタンダードとして用いられている。

現在行われているトクホ食品認定のための酸産生性検定の要件を表１に掲げたが、これは国際トゥースフレンドリー協会の検定要件とほぼ同等**である。

表1　厚生労働省特定保健用食品における酸産生性測定法の実際（検定要件）

- **被験者4名以上**
- **pH電極内蔵法による酸産生性試験**
  あるいは同等の方法でpH＞5.7（30分）
- **非水溶性グルカン合成試験**

以上のことから、現在行われている検定システムは、「Evidence-based prevention」の立場から、う蝕予防食品を検定する目的に最適であると考えられる。

*　米国ではう蝕予防食品検定は行われてない。

**　国際トゥースフレンドリー協会の検定要件には「非水溶性グルカン合成試験」は含まれていない。また、pH 低下基準は pH ≧5.7（30 分）と若干異なる。

1)-2　運用性について

現在のシステムは、科学的信頼性が高いという優れた長所をもつが、以下のような欠点をもつことが明らかになった。

まず、①被験者の確保が容易ではないこと、②被験者の負担が大きいこと、③測定者の負担が大きいこと、④測定装置が高価であること等、「様々な面でコストが高いこと」が欠点として挙げられる。さらに、⑤現在の大学研究室という環境では検定業務が研究実績として評価されにくい上、検定専任者のポストの確保が難しいため、「検定業務の効率が悪いこと」が欠点として挙げられる。

欠点⑤「検定業務の効率が悪いこと」を克服するためには、専門の検定機関の設置等が必要であると考えられる。歯科以外の健康食品の検定に関しては、民間の検定会社等に検定を委ねることで効率化が図れる可能性が考えられるが、歯科健康食品に対する検定が特殊であることや歯科食品市場がまだあまり大きくないこと等のため、現時点では、民間の検定会社等に委ねることは困難であると考えられる。さらに、検定の中立性・公平性を慮れば、公的機関に準ずる検定機関の設立がより望ましいものと思われる。

一方、欠点①～④「コストが高いこと」については、基本的に信頼性の高い検定を行うためにはやむを得ないことと思われる。①被験者の確保が容易ではないこと及び③測定者の負担が大きいことは、大学の一研究室という環境で検定業務を行うために生ずる問題であり、上記のような検定機関が設立されれば解決可能であると考えられる。

現在、*In vitro* 測定法や人工口腔装置等の代替法が開発されつつあり、これらを用いることでコストを下げることが可能と思われる。しかし、代替法の信頼性を保障するための研究はまだ始まったばかりであり、また代替法が一般化しても、信頼性が高い検定法としての電極内臓法は、常に使用できるようにしておくことが必要と思われる。

2) 酸産生性検定の位置付けについて

今後、健康食品評価指標が増加するに従って、これまで先行して行われてきた酸産生性評価指標はその位置付けが変化して行くものと思われる。とくに、歯科健康食品評価が単に「酸を産生しない」というような所謂「レス食品」ではなく、より積極的な機能をもつ「機能性食

品」を評価の対象とするようになることで、歯科健康食品の中での酸産生性評価は、これまでのような十分条件ではなくなり、機能性食品検定へ至るための必要条件、すなわち「歯表面脱灰を起こさない安全な食品」を選定する「前段階検定」という位置付けへ変化して行くものと思われる。

一方、健康食品全体を対象とした場合、口腔から食品が摂取される以上、歯表面の脱灰を引き起こすおそれのある酸産生性食品はその危険性を明示されなければならないと考えられる。このような観点から、酸産生性検定は、歯科健康食品に対する「前段階検定」だけではなく、健康食品全体に対しても行われるべき「前段階検定」ではないかと思われる。

酸産生性という評価指標は、新たな評価指標とは異なり、これまでに十分な科学的裏付けがなされていることから、その信頼性は高いと考えられる。従って、酸産生性検定に関してはより簡略化した検定手順を考慮してもよいのかもしれない。例えば、表1の検定要件に記された被験者の数を、より少なくすることも可能であると思われる。とくに健康食品の前段階検定として捉えるならば、種々のコスト削減の観点から、簡略化は必須であろう。

さらに、歯表面の脱灰を引き起こすおそれのある食品は酸産生性食品（＝プラーク細菌によって発酵され酸を産生する）だけではなく、自らが酸性である食品も含む。酸性を示す食品を摂取することによって歯表面の脱灰を来すことを「酸蝕」あるいは「酸蝕症」というが、酸産生性だけではなく酸蝕性を検定し評価することが、今後の重要な課題であると考えられる。

1－E．結　　論

プラークpHテレメトリー法（電極内蔵法）を食品酸産生能測定法として採用している東北大学大学院歯学研究科の食品酸産生性検定システムは、信頼性は高いが、運用性としては「コストが高い」「検定業務の効率が悪い」という欠点をもつことが分かった。

しかし、これらの欠点は、大学の一研究室で検定業務が行われているために生ずるものと考えられ、検定業務を専門的に行う検定機関の設置等によって克服できるものと考えられた。

また、健康食品検定における酸産生性検定は、今後、健康食品の機能性の評価指標が増えるに従って、より相対化され、歯科健康食品の前段階検定へ変質して行くものと思われた。しかし、口腔から食品が摂取される以上、歯表面を脱灰する可能性のある酸産生性食品はその危険性を明示する必要があると考えられることから、健康食品全体に対して、前段階検定としての酸産生性検定が重要になるものと思われた。

さらに、歯表面脱灰の原因となるのは酸産生性食品だけではなく酸蝕性食品も含まれることから、今後、酸蝕性食品についての検討が必要であると考えられる。

2．健康食品のバイオフィルム形成に及ぼす影響に関する検討

（DNA マイクロアレイによる口腔細菌叢の検討）

2－A．研究目的

う蝕や歯周病などの口腔内細菌性疾患の発症は、口腔表面に形成されるバイオフィルム微生物叢によって惹起される。口腔バイオフィルムは、膨大な数と種類の微生物によって構成される微生物叢生態系であり、この微生物叢をコントロールすることがこれらの疾患を予防する一方法となる。

昨年の研究で、口腔微生物特異性が高い DNA プローブとそれを用いた目視可能な判別システムを確立した。そこで本年度は、これを応用し、口腔微生物叢解析用マイクロアレイ・プロトタイプの作成を試みた。

2－B．研究方法

昨年度の研究で作成された、16S ribosomal RNA gene に基づく口腔微生物 24 種の微生物種特異的 DNA プローブを特殊紙上に固定し、DNA マイクロアレイを試作した。

次いで、標準口腔微生物株および臨床サンプル（プラーク）を用いて、微生物種特異性ならびに検出感度を検討した。

2－C．研究結果
2－D．考　　察

完成した DNA マイクロアレイ・プロトタイプに対して、標準口腔微生物株を用い、微生物種検出特異性ならびに検出感度を検討し、概ね良好な結果が得られた。また、DNA マイクロアレイ・プロトタイプのスポット検出法として採用した色素法は、容易に肉眼で判定できることが明らかになった。

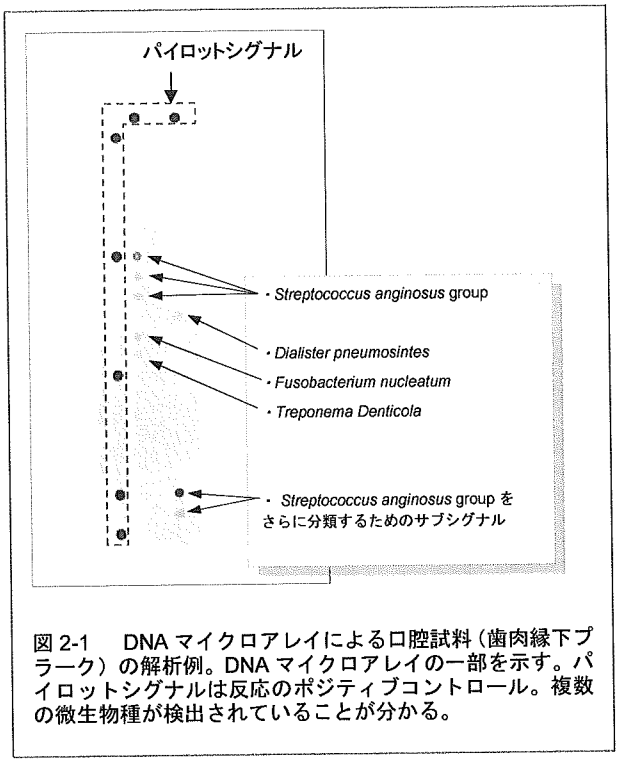

図 2-1　DNA マイクロアレイによる口腔試料（歯肉縁下プラーク）の解析例。DNA マイクロアレイの一部を示す。パイロットシグナルは反応のポジティブコントロール。複数の微生物種が検出されていることが分かる。

さらに、臨床試料（歯肉縁下プラーク）を用いて検討したところ、臨床試料で測定可能であることを確認できた（図 2-1）。現在、実用化に向けた反応最適条件の検討、および定量結果との整合性を検討しているところである。さらに、複数の口腔疾患に対応できる DNA マイクロアレイの作成を目指すために、対象とする口腔微生物種の数を増やすこととした。現在、微生物種の選択を終え、これら微生物種の種特異的 DNA プローブを作成中である。

なお、本 DNA マイクロアレイおよび検出装置については、現在「口腔バイオフィルム微生物叢 DNA マイクロアレイ解析システム」として、共同研究企業とともに特許取得の準備を進めている。

2－E．結　　論

以上のことから、口腔微生物特異性が高くかつ肉眼で判別可能な口腔バイオフィルム微生物叢解析用DNAマイクロアレイ・プロトタイプが完成した。

さらに、微生物種を増やすことで、複数の口腔疾患に対応できるDNAマイクロアレイの作成を目指すこととした。

F．健康危険情報：特になし

G．研究発表（囲みは関連の強いもの）

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H. 知的財産権の出願・登録状況

1. 特許出願（予定）

(1) 口腔バイオフィルム微生物叢DNAマイクロアレイ解析システム

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Characteristic Intestinal Microflora of Specific Pathogen-Free Mice Bred in Two Different Colonies and their Influence on Postnatal Murine Immunocyte Profiles

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Abstract: *Cecal microflora of BALB/c mice originating from two different SPF-breeding colonies were compared. The analysis of cultivable bacteria in the ceca showed significantly higher numbers of total bacteria in BALB/cCrSlc (SLC mice) than in BALB/cA Jcl (JCL mice) ($p<0.05$), which were mainly based on higher numbers and occurrence of Peptococaceae. Bifidobacteria were detected only in SLC mice. Feeding an oligosaccharide, raffinose, to the mice also induced different shifts in the composition of cecal microflora and the concentration of cecal organic acids. In the second experiment, hysterectomy-derived (HD) SLC mice were fostered to SPF lactating SLC mothers, or SPF lactating JCL mice, together with the mother's own natural birth (NB) pups in each isolator. HD mice fostered to SLC-mothers showed significantly higher percentages of T-cell receptor αβ cells expressing a CD8α homodimer ($p<0.05$) and a CD8αβ heterodimer ($p<0.001$) in the intraepithelial lymphocytes (IEL) compared with HD mice fostered to JCL-mothers. IEL profiles of HD mice corresponded well to those of NB mice that were breast-fed by the same mothers. Differences in the ratio of B220$^+$cells to Thy1.2$^+$cells in the splenocytes were also observed as a trend between both HD mice fostered to SLC or JCL mothers ($p=0.06$). These results suggest that postnatal colonization of various characteristic intestinal microflora derived from SPF-breeding colonies results in differences in development of lymphocyte populations in the intestinal and systemic organs of mice.*

Key words: *immunity, intestinal microflora, intraepithelial lymphocytes, SPF mice*

Introduction

It is considered that postnatal formation of intestinal microflora of mammals is influenced by bacterial contamination from the mother during lactation [5, 8]. It has also been reported that the composition of intestinal microflora of adult mice was based on genetic factors in each mouse strain [7] including levels of major histocompatibility complex [11], and was not affected very much by bacterial contamination from

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the mothers [7]. Thus if mice, particularly inbred mice, are bred in barrier-sustained colonies, their intestinal microflora should be strongly affected by their mother's microflora because they are strictly isolated from the external environment. It is possible that the characteristic composition of microflora in each breeding colony might have different effects on characteristics of SPF mice. Microbes have various characteristics related to microbial metabolism, immunogens and pathogenicity in hosts. However, there are few reports showing differences in intestinal microflora of mice in SPF-breeding colonies, and also the influence of these differences on characteristics of SPF mice. In order to clarify the situation, we focused on whether different SPF-intestinal microflora affect development of immune organs because the immune system is susceptible to colonization of intestinal microbes. For example, germ-free animals are well known to show immunological underdevelopment of systemic and intestinal immune organs, such as the spleen, lymph nodes [4], Peyer's patches [10] and intraepithelial lymphocytes (IEL) [2, 14], compared with conventional animals.

In this study, we compared the cecal microflora of two BALB/c mice from different breeding colonies and also examined the composition of cecal microflora and the concentration of cecal organic acids after feeding indigestible oligosaccharide to both groups of mice. Lastly, we demonstrated that postnatal association with these 2 kinds of SPF-microflora in hysterectomy-derived mice induces development of different immunocyte profiles in the hosts.

Materials and Methods

Animals

SPF animals, female BALB/cCrSlc mice (SLC mice), female BALB/cAJcl mice (JCL mice), and pregnant mice, were purchased from Japan SLC (Hamamatsu, Japan) and CLEA Japan (Tokyo, Japan). The mice used for analysis of intestinal microflora were housed in plastic cages at 22 ± 2°C with a 12 h light-dark cycle under conventional conditions. The mice used for foster-nursing experiments were housed in vinyl isolators sterilized with 2% peracetic acid. Metal cages and water were sterilized by autoclaving at 121°C for 70 min. The isolators were placed in a room with a controlled 12 h light dark cycle at 24 ± 1°C with a relative humidity of 55 ± 5%. All mice were given sterilized diet and water *ad libitum*. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals of The University of Tokyo.

Diets

The purified basal diet was composed of (g/kg) casein 200.0, maize starch 481.7, α,β-starch 90.0, sucrose 50.0, cellulose 50.0, soyabean oil 60.0, mineral mixture (AIN-76) 50.0, vitamin mixture (AIN-76) 13.0, choline chloride 2.3, and methionine 3.0 (1). The oligosaccharide diet was prepared by adding raffinose (Nippon Beet Sugar Mfg, Tokyo, Japan, purity > 995 g/kg DM) to the basal diet (50 g/kg diet) instead of maize starch. The diets were pelletized and vacuum-sealed in plastic bags by Funabashi Farm (Chiba, Japan), and were sterilized by γ-irradiation at 10 kGy.

Oligosaccharide feeding experiment

BALB/cCrSlc mice and BALB/cAJcl (6 weeks of age) were randomly allocated to two groups (n=5 per group, total 4 groups). Each group was fed the basal diet or the oligosaccharide diet for 2 weeks. After the mice were sacrificed by cervical dislocation, ceca removed from the mice were each subjected to analysis of intestinal microflora and organic acids.

Foster-nursing experiment: association with different SPF microflora

Ten pregnant BALB/cCrSlc mice were maintained under SPF conditions. After the pups were removed from the pregnant mice by hysterectomy, the pups were randomly fostered to BALB/cCrSlc lactating mothers (n=5) or BALB/cAJcl lactating mothers (n=5), together with the mother's own natural birth pups in each vinyl isolator. After both the hysterectomy-derived (HD) and natural birth (NB) pups were weaned at 4 weeks of age, they were kept in the same isolators until 6 weeks of age. Thereafter, HD and NB mice were sacrificed by cervical dislocation, and the small intestine, spleen and mesenteric lymph nodes (MLN) removed from these mice were each subjected to flow cytometric analysis.

Analysis of intestinal microflora

Bacteriological analysis of mice cecal contents was carried out according to the method of Mitsuoka [9]. Briefly, fresh samples were immediately diluted in 10-

fold steps with anaerobic phosphate buffer and 0.05 ml of each dilution was inoculated on two non-selective media (BL and TS agar) and three selective media (mLBS, DHL and TATAC agar). BL and mLBS agar plates were incubated at 37°C for 48 h in an anaerobic steel wool jar filled with oxygen-free CO_2, and TS, DHL and TATAC agars were incubated for 24–48 h aerobically. Bacterial groups were identified using colony and cell morphology, Gram staining, spore formation and aerobic growth.

Determination of cecal organic acids

Cecal contents were weighed and homogenized with a 20-fold volume of 0.2 N HCl. Pyroglutamic acid was used as the internal standard. Organic acids in the cecal contents were determined by high performance liquid chromatography using the postcolumn method with bromothymol blue as pH indicator (wave length for detection; 445 nm, column; RSpak KC-811, Showa Denko K.K., Tokyo, Japan).

Lymphocyte preparation

IEL were prepared as previously described [6]. In brief, contents of the small intestines of mice were thoroughly washed out with Hank's balanced salt solution (HBSS: Gibco BRL, Gaithersburg, MD). Each intestine was inverted, and individually transferred to a 50-ml conical tube containing 45 ml of HBSS supplemented with 5% (v/v) fetal calf serum (Sigma-Aldrich, Mo, USA). The tubes were shaken at 150 rpm in the horizontal position for 45 min at 37°C, and then were shaken several times by hand. Each resultant cell suspension was collected and filtered through a glass-wool column. Subsequently, the cells were suspended in 30% Percoll (Pharmacia Biotech, Uppsala, Sweden) solution and centrifuged at 400 × g for 20 min. Cells pelleted at the bottom of the tube were applied to Percoll discontinuous density gradient centrifugation. IEL were recovered at the 44 to 70% Percoll interface. Single-cell suspensions of splenocytes and MLN cells were prepared by mashing the organ with the end of a syringe, and passing each cell suspension through a polyester mesh.

Flow cytometry

Lymphocytes (5×10^5 cells) were incubated with fluorochrome-labeled or biotinylated monoclonal antibodies (mAb) for 20 min in ice water after blocking Fc receptors with anti CD16/32 mAb (2.4G2; PharMingen, San Diego, CA). If necessary, fluorochrome-labeled streptavidins were used as the second antibodies. After staining, the cells were washed with HBSS containing 1% (w/v) fetal calf serum and 0.01% (w/v) sodium azide. Cytofluorometric analysis was performed by flow cytometry (FACSort, Becton Dickinson, Franklin Lakes, NJ). The following mAb were used: biotin-anti-TCRβ mAb (H57-597; PharMingen), fluorescein isothiocyanate (FITC)-anti-TCRγ mAb (GL-3; Cedarlane, Hornby, Ontario, Canada), biotin-anti-TCRγ mAb (GL-3, Cedarlane), FITC-anti-CD4 mAb (H129.12; PharMingen), phycoerythrin (PE)-anti-CD8α mAb (53–6.7; Gibco BRL), FITC-anti-CD8β mAb (Y8.77; Seikagaku-Kogyo, Tokyo, Japan), PE-anti-B220 mAb (RA3–6B2, Gibco BRL), FITC-anti-Thy1.2 mAb (30-H12, PharMingen), FITC-streptavidin (Gibco BRL), PE-streptavidin (Gibco BRL), and RED 670-streptavidin (Gibco BRL); all were purchased from the sources indicated.

Statistical analysis

Results were expressed as mean values with standard deviations. Differences in bacterial counts and organic acid concentrations in the ceca of mice were analyzed using Scheffe's F post-hoc test, and differences in occurrences of microbes were analyzed using Fisher's exact probability test. Differences in lymphocyte profiles of mice were analyzed using Student's *t* test.

Results

Intestinal microflora of mice bred in different colonies

Differences in cecal microflora between SLC mice and JCL mice were compared in terms of cultivable bacteria (Table 1). SLC mice showed significantly higher numbers of total bacteria than JCL mice ($p<0.05$). In particular, higher numbers and significantly higher occurrences of Peptococaceae in SLC mice were observed compared with those in JCL mice ($p<0.05$). The percentage of Peptococaceae to total bacteria was 52 ± 16% in SLC mice, while that in JCL mice was below 4%. Bifidobacteria were detected only in SLC mice. There were no differences between groups in numbers of facultative aerobes such as Lactobacilli, Enterobacteriaceae and Streptococaceae. Feeding of the oligosaccharide diet

Table 1. Comparison of cecal microflora of BALB/cAJcl and BALBc/CrSlc fed basal diet or oligosaccharide diet

	Basal diet				Oligosaccharide diet			
	BALB/cAJcl		BALB/cCrSlc		BALB/cAJcl		BALB/cCrSlc	
Total bacteria	9.1 ± 0.2	(100)[a)]	9.6 ± 0.3*	(100)	9.5 ± 0.1	(100)	9.9 ± 0.3	(100)
Bacteroidaceae	8.8 ± 0.2	(100)	9.1 ± 0.2	(100)	9.4 ± 0.2#	(100)	9.3 ± 0.4	(100)
Bifidobacteria		(0)	7.2	(40)		(0)	7.8 ± 0.4	(100)
Eubacteria	7.9	(40)	7.9	(40)	8.3	(20)	8.6 ± 0.6	(80)
Peptococaceae	7.6	(20)	9.3 ± 0.4	(100)*	6.6	(20)	9.6 ± 0.2	(100)
Clostridia		(0)	7.2	(40)	8.1	(40)	7.8 ± 0.4	(100)
Fusiform bacteria		(0)		(0)	8.0 ± 0.2	(60)#		(0)
Curved rods	7.6	(20)		(0)	7.6	(40)		(0)
Lactobacilli	8.6 ± 0.4	(100)	8.5 ± 0.2	(100)	8.0 ± 0.6	(100)	8.3 ± 0.4	(100)
Enterobacteriaceae	5.4 ± 0.5	(100)	5.6 ± 0.4	(100)	4.9 ± 0.9	(100)	4.4 ± 0.2#	(100)
Streptococaceae	6.6 ± 0.3	(100)	6.5 ± 0.5	(100)	6.6 ± 0.0	(100)	6.5 ± 0.7	(100)

The mice were fed purified basal diet or oligosaccharide diet (containing raffinose at 50 g/kg) for 2 weeks. a) Values are expressed as mean ± S.D. of bacterial counts (log no./g cecal contents). Figures in parentheses refer to frequency of occurrence (%). Significant difference * ($p<0.05$) between BALB/cA and BALB/cCr fed basal diet, # ($p<0.05$) between basal diet and oligosaccharide diet within the same mouse strain.

Table 2. Cecal organic acids of BALB/cAJcl and BALB/cCRSlc fed basal diet or oligosaccharide diet

	Basal diet		Oligosaccharide diet	
	BALB/cAJcl	BALB/cCrSlc	BALB/cAJcl	BALB/cCrSlc
Acetic acid	25.4 ± 7.5	23.1 ± 7.8	25.5 ± 8.8	29.2 ± 6.4
Propionic acid	3.3 ± 0.7	3.7 ± 0.8	4.8 ± 2.0	8.0 ± 1.9#
n-Butyric acid	2.7 ± 0.7	3.1 ± 1.6	4.7 ± 3.2	9.2 ± 3.0#
Formic acid	0.1 ± 0.1	1.1 ± 0.4*	0.0 ± 0.0	0.2 ± 0.3#
Succinic acid	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.4	0.2 ± 0.2
Lactic acid	0.3 ± 0.2	0.0 ± 0.0	0.2 ± 0.2	1.2 ± 1.1#

Values are expressed as mean ± S.D. of organic acids (μmol/g cecal contents) of mice. * and #; see footnotes of Table 1.

(50 g raffinose/kg diet) to JCL mice for 2 weeks significantly increased numbers of Bacteroidaceae ($p<0.05$) and occurrence of fusiform bacteria ($p<0.05$). In contrast, SLC mice fed oligosaccharide diet showed increasing tendencies in the numbers or occurrences of various strict anaerobes such as Bacteroidaceae, bifidobacteria, eubacteria, Peptococaceae and clostridia, and a significant decrease in the number of Enterobacteriaceae ($p<0.05$). On the other hand, the organic acid concentration in the ceca of mice fed a basal diet showed a significant difference only in formic acid concentration between SLC and JCL mice (Table 2). There were no significant differences in cecal organic acids of JCL-mice between mice fed the basal diet and the oligosaccharide diet. However, the oligosaccharide diet fed to SLC mice induced significant increases of cecal propionic, butyric and lactic acid concentrations compared with those fed the basal diet ($p<0.05$).

Effect of postnatal association with different SPF microflora on lymphocyte profiles of the mice

HD mice were fostered to SPF lactating SLC mothers or SPF lactating JCL mothers in each isolator together with NB pups. With this method, intestinal microflora of HD mice should have been transferred from foster mothers. There were marked differences in the IEL populations between SLC-NB mice and JCL-NB mice (Fig. 1). The SLC-NB mice showed higher percentages of TCR$\alpha\beta$ IEL and lower percentages of TCR$\gamma\delta$ IEL than the JCL-NB mice. The high percent-

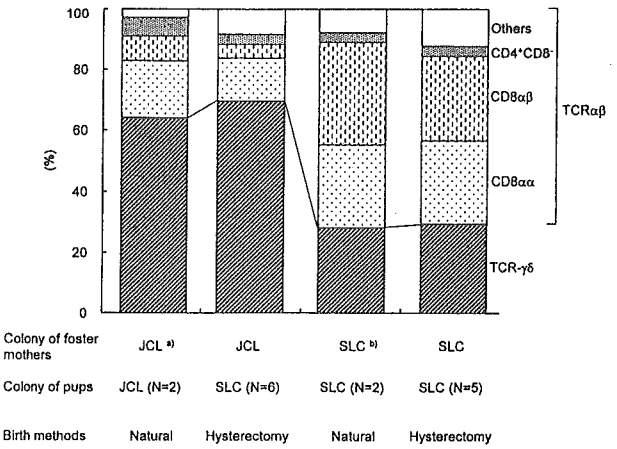

Fig. 1. Comparison of intraepithelial lymphocyte profiles among natural birth and hysterectomy-derived BALB/c mice fostered to lactating mothers from different colonies. The hysterectomy-derived (HD) SLC mice were fostered to SPF lactating mothers together with the mother's own natural birth (NB) pups until 4 weeks of age. Data show means of each population in intraepithelial lymphocytes of NB and HB mice at 6 weeks of age. a) JCL; BALB/cA Jcl. b) SLC; BALB/cCrSlc.

age of TCR$\alpha\beta$ IEL in the SLC-NB mice consisted of increased percentages of both CD8$\alpha\alpha^+$ cells and CD8$\alpha\beta^+$ cells. The percentages of CD8$\alpha\beta^+$ cells and CD8$\alpha\alpha^+$ cells in SLC-NB mice were 4-fold and 2-fold higher, respectively, than those in JCL-NB mice. There were few differences in the percentages of CD4$^+$ TCR$\alpha\beta$ IEL between SLC-NB mice and JCL-NB mice. On the other hand, IEL profiles of HD mice show good agreement with those of NB mice that were breast-fed by the same mothers. HD mice fostered to SLC-mothers showed significantly higher percentages of TCR$\alpha\beta$ IEL expressing CD8$\alpha\alpha$ ($p<0.05$) and CD8$\alpha\beta$ ($p<0.001$) compared with HD mice fostered to JCL-mothers. Therefore, the ratio of TCR$\alpha\beta$ IEL to TCR$\gamma\delta$ IEL was significantly higher in SLC-fostered HD mice compared with JCL-fostered HD mice ($p<0.05$: SLC-fostered HD mice, 2.4 ± 0.2; JCL-fostered HD mice, 0.4 ± 0.1). Moreover, the ratio of B220$^+$cells to Thy1.2$^+$cells in the spleen of SLC-fostered HD mice tended to be higher than that in JCL-fostered HD mice (Table 3).

Table 3. Ratio of B220$^+$ cells to Thy1.2$^+$ cells in the spleen and mesenteric lymph nodes of hysterectomy-derived mice fostered to lactating mothers for different colonies

	Foster mother		
	JCL[a]	SLC[b]	p
Spleen	1.88 ± 0.39	2.33 ± 0.06	0.06
Mesenteric lymph nodes	0.42 ± 0.09	0.52 ± 0.07	0.11

Ratio of B220$^+$ cells to Thy1.2$^+$ cells in the spleen and mesenteric lymph nodes of hysterectomy-derived mice shown in Fig. 1 (means ± SD, n=4) p values were calculated using Student's t test. a, b) see footnotes in Fig. 1.

Discussion

It is possible that the compositions of intestinal microflora in SPF experimental animals might differ among breeding colonies, and this difference might have some effect on development of immune organs in the animals. In order to prove this hypothesis, cecal microflora of different colonies of BALB/c mice, BALB/cCrSlc and BALB/cAJcl, were compared. An analysis of cultivable bacteria in the ceca showed higher numbers and occurrences of Peptococaceae in SLC mice than in JCL mice. Bifidobacteria were also detected only in SLC mice. These results show that there was a major difference in composition of cecal anaerobes between JCL and SLC BALB/c mice. Raffinose is known to be a fermentable indigestible oligosaccharide and growth factor of bifidobacteria *in vitro* [15] and in humans *in vivo* [3]. Feeding this sugar to mice also induced different changes of various bacterial counts between the two mice colonies. Analysis of changes in cecal organic acids by oligosaccharide feeding also gave different results: a significant increase of some organic acids in SLC mice but no changes in JCL mice. The difference in metabolism of orally fed oligosaccharide by indigenous microflora suggests that not only cultivable bacterial counts in the intestine but also essential microbes composing microflora differed in SLC and JCL mice.

In the second experiment, HD mice were fostered to SPF lactating SLC mothers or SPF lactating JCL mothers together with the mother's NB pups. This method resulted in association of HD and NB mice with the intestinal microflora of the foster mother in the isolator. HD and NB mice acquired identical intestinal microflora because the BALB/cCr and BALB/cA mice used in the present study have identical genetic backgrounds. SLC-NB mice and SLC-fostered HD mice showed significantly higher percentages of TCR$\alpha\beta$ cells

expressing CD8 molecules in IEL compared with those of JCL-NB and JCL-fostered HD mice. This result suggests that SLC-flora induce expansion of $CD8^+$ TCR$\alpha\beta$ IEL more strongly than JCL-flora. Moreover, the ratios of $B220^+$cells (B-cell marker) to $Thy1.2^+$cells (T-cell marker) in the splenocytes also differed between SLC-fostered HD mice and JCL-fostered HD mice.

It is known that the number of TCR$\alpha\beta$ IEL in germ-free mice is greatly reduced compared with conventional mice [2, 14]. The ratios of TCR$\alpha\beta$ cells to TCR$\gamma\delta$ cells in the IEL of the JCL-fostered HD mice in our results showed good agreement with those of germ-free mice reported previously (JCL-fostered HD mice, 0.4 ± 0.1; germ-free mice, 0.39 as reported in Reference No.13). These results suggest that JCL-flora might lack microbes able to induce expansion of $CD8^+$ TCR$\alpha\beta$ IEL. It has been reported that mono-association with segmented filamentous bacteria (SFB) in germ-free mice induced marked expansion of $CD8^+$ TCR$\alpha\beta$ IEL in the small intestine from germ-free levels to conventional levels [13]. In the present study, we were not able to clarify whether SFB colonized the intestinal lumens of JCL and SLC mice because SFB is a non-cultivable bacteria [12].

In conclusion, we showed that BALB/c mice bred in two different colonies have essentially different compositions of intestinal microflora in terms of not only bacterial counts but also metabolic responses to oligosaccharides. Moreover, postnatal association with each microflora in HD mice resulted in different development of lymphocyte populations in the intestinal and systemic immune systems. Specific indigenous microflora of each breeding colony might affect not only development of the immune system but also systemic metabolism and pathology. We consider that investigators should pay more attention to the possibility that results obtained from animal experiments might be influenced by characteristic microflora of each breeding colony.

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Effect of specific antigen stimulation on intraepithelial lymphocyte migration to small intestinal mucosa

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Summary

Migration of intraepithelial lymphocytes (IELs) into intestinal epithelium is not yet well understood. We established an IEL-cell line from ovalbumin (OVA) 23–3 transgenic (Tg) mice and investigated the effect of antigen stimulation on the dynamic process of IEL migration into small intestinal mucosa. The cell line was a T cell receptor (TCR) $\alpha\beta^+$ $CD4^+$ $CD8^-$ phenotype, expressing $\alpha E\beta 7$ integrin in 90% of cells. Under intravital microscopy, the lined IELs adhered selectively to the microvessels of the intestinal villus tip of the Tg mice. The accumulation of IELs was significantly inhibited by an antibody against β7-integrin and MAdCAM-1. When IELs were stimulated with OVA, the accumulation was attenuated compared to that of resting cells, with decreased expression of $\alpha E\beta 7$ integrin. In Tg mice fed with OVA, the number of IELs which migrated in the villus mucosa was significantly smaller than in the non-fed controls. The preferential migratory capacity of IELs to villus mucosa may be altered by specific antigen stimulations.

Keywords: adhesion molecules, cell trafficking, intraepithelial lymphocytes, ovalbumin, small intestine

Introduction

The intestinal epithelium is a region constantly exposed to intraluminal substances, such as intestinal flora and food antigens. It has been suggested that the functions of intraepithelial lymphocytes (IELs) are first-stage protection and maintenance of the epithelial layer. IELs consist of both αβ and γδ T cell receptor (TCR)-bearing cells with phenotypic and functional features distinct from those of cells in the peripheral lymphoid tissue [1]. Indeed, peripheral T cells and IELs have many different characteristics; for example, concanavalin A (ConA), which is mitogenic to peripheral T cells, cannot stimulate IELs [2]. These lymphocytes, mainly $CD8^+$ T cells, are largely unresponsive to proliferative signals mediated via conventional stimulation of the CD3–TCR complex [3]. Most IELs express characteristic integrin $\alpha E\beta 7$, which is hardly observed in peripheral T cells [4,5].

These T cells have diverse sources of origin: it is known that most peripheral T cells differentiate in the thymus, but a large population of IELs differentiate extrathymically. Some IELs may arise *in situ* in the gut epithelium [6]. Some may develop in the thymus, and yet others come from extraintestinal, extrathymic sources, and these lymphocytes reach the intestine. IEL precursors in the blood may gain access to the mucosal sites. It has been speculated that the γδ T cells present in the vaginal epithelium originate from the peripheral lymphoid organs [7]. There have been reports that injected peripheral T cells, under adequate conditions, can fill the epithelial compartments and acquire the characteristics of IELs [6,8]. However, there has been little information about the exact route and mechanisms by which IELs reach the microvessels of intestinal villi before they gain access to the epithelium.

There is evidence that the homing behaviour of lymphocytes can be altered profoundly by activation and differentiation. The migration properties of activated lymphocytes appear to be both more selective and more diverse than those of naive lymphocytes. Some migration properties of 'memory' lymphocytes resemble more closely those of activated lymphocytes [9–11]. Most memory and effector lymphocytes probably traffic through lymphoid organs, but unlike naive cells, they can also access and recirculate through extralymphoid immune effector sites such as the intestinal mucosa or inflamed skin and joints [11–13]. In humans, for example, CD4 cells that express both CLA and L-selectin preferentially accumulate in inflamed skin [14]. However, the effect of antigen-specific activation of IELs on their

trafficking within microvessels supplying blood to the gut mucosa remains unclear.

Much remains to be clarified with regard to the functions and homing patterns of IELs and their molecular basis. One reason for the difficulty in studying IELs is the lack of appropriate cell lines established from these populations. The difficulty of establishing IEL cell lines is due to the poor proliferative capacity and a lack of knowledge about their adequate ligands. In the present study, we have established an antigen-specific IEL cell line from ovalbumin (OVA)23-3 mice expressing a transgenic (Tg) TCR$\alpha\beta$ specific to OVA323-339 [15]. The majority of T cells in these mice expressed a TCR specific to this epitope, which gave us an advantage in establishing the antigen-specific cell line. Using this cell line, we analysed the functional characteristics of cells, such as their cytokine production and antigen-specific proliferation.

Recent *in situ* microscopy experiments with intestinal mucosa have demonstrated that lymphocyte homing involves organ-specific multi-step cascades of adhesion and signalling events in specialized blood vessels, termed high endothelial venules (HEV), as well as in the villus microvessels [16–18]. In this study, using the established IEL cell line we carried out an intravital microscopic procedure to monitor the dynamic process of lymphocyte migration in order to (1) investigate whether adhesion of IEL cells occurs in the villus mucosa of the small intestine and, if it does, examine a possible contribution of various adhesion molecules to this IEL–endothelial cell adhesive interaction, and (2) compare how recruitment of naive and antigen-specifically stimulated IEL cells differs in the villus mucosa of the small intestine.

Methods

OVA23-3 Tg mice and isolation of IEL

The process of establishing the OVA23-3 Tg mouse line we used has been described previously [15]. The mice carried a gene encoding TCR $\alpha\beta$ (Vα3.1/Vβ15) derived from an OVA-specific CD4$^+$ T cell clone, 7-3-7. The animals were housed and bred within animal facilities at the University of Tokyo. We obtained transgenic mice from the F_1 generation of a cross between BALB/c (Clea Japan, Inc., Tokyo, Japan) and heterozygous transgenic mice. The care and use of laboratory animals were in accordance with the guidelines of the National Institute of Health.

IELs were isolated from Tg mice of both sexes, 8–24 weeks of age, by using modified procedures as described previously [19]. Briefly, an inverted intestine was cut into four segments and the segments were transferred into a 50-ml conical tube containing 45 ml of 5% fetal calf serum (FCS) in Ca^2, Mg^2-free Hanks's balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY, USA). The tube was shaken in an orbital shaker at 150 r.p.m. in the horizontal position for 45 min at 37°C. Cell suspensions were collected and passed through a glass-wool column to remove cell debris and adherent cells. Subsequently, the cells were suspended in 30% (wt/vol) Percoll (Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 20 min at 600 *g*. After the centrifugation, the cells at the bottom of the solution were subjected to Percoll discontinuous-gradient centrifugation and IELs were recovered at the interface of 44% and 70% Percoll (>95% were CD3$^+$). The obtained cell suspensions were washed and stored on ice in RPMI (pH 7·4) with 5% FCS until used.

Establishment of IEL cell line and antigen stimulation

IELs from OVA23-3 mice (10^6 cells/ml) were stimulated every week with mitomycin C-treated CD4$^-$ BALB/c splenocytes (2×10^6 cells/ml) in a culture medium with 5 mM OVA323-339 peptide and a 10% culture supernatant of ConA-stimulated rat splenocytes [20]. The method of mitomycin C treatment was as follows: 10^7 splenocytes/ml were incubated with 50 mg/ml of mitomycin C (Sigma) for 45 min at 37°C and washed with an RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) three times. The medium for the cell culture was RPMI-1640 containing 100 U/ml penicillin, 100 mg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 10% FCS (Cansera International, Rexdale, Canada). Several weeks after the initiation of the culture, the growing cells were expanded and then an antigen-specific IEL cell line from the OVA23-3 mice was established. The IEL cell line was induced to rest by changing only the culture medium without antigen-presenting cells once a week for 4 weeks after the final antigen stimulation.

An antigen-stimulated IEL cell line was obtained by removing CD4$^-$ splenocytes by magnetic cell sorting (MACS) soon after the final antigen stimulation. In brief, the IELs (1×10^7) with CD4$^-$ splenocytes were suspended in 90 µl of phosphate buffered saline (PBS) containing 0·5% bovine serum albumin and 5 mM ethylenediaminetetra acetic acid (EDTA) and incubated in 10 µl of antimouse CD4 (L3T4)-labelled MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min at 6°C. After that, the treated IELs were passed through a separation column (type MS, Miltenyi Biotech) placed in the magnetic field of a MACS separator. The magnetically labelled CD4$^+$ IEL cells were retained in the column, while the unlabelled CD4$^-$ splenocytes ran through. After removing the column from the magnetic field, the retained CD4$^+$ IEL cells could be eluted as an antigen-stimulated IEL cell line. The CD4$^+$ IEL cells were washed and, until used, resuspended in RPMI-1640 with 5% FCS stored on ice.

Cell proliferation and cytokine production assay

The cell line was plated in 96-well plates at 1 or 2×10^5 cells/well with OVA or ConA (10 mg/ml) and at 4×10^5 cells/well with antigen-presenting cells (APC) (mitomycin C-treated

BALB/c splenocytes) in a total volume of 200 ml. After 24 and 48 h, 0·5 mCi of [^{3}H]thymidine was added to each well. The cells were harvested 20 h later and the [^{3}H]thymidine incorporation was measured by scintillation counting. Cytokines in the culture supernatants were detected using a two-site sandwich enzyme-linked immunosorbent assay (ELISA) as described previously [21]. Briefly, for assays of interleukin (IL)-4, IL-5 and interferon (IFN)-γ, Maxisorp immunoplates (Nunc Roskilde, Denmark) were coated with a rat antimouse IL-4 (BVD4–1D11, PharMingen, San Diego, CA, USA), a rat antimouse IL-5 and a rat antimouse IFN-γ antibody (XMG1.2). This was followed by incubation with alkaline phosphatase-streptavidin. A substrate (p-nitrophenyl phosphate) was added and the colour development was stopped by the addition of 5 N NaOH. The absorbance was determined at 405 nm.

Flow cytometry

Cells (2×10^5/sample) were washed with HBSS containing 5% FCS and 0·2% NaN_3 (the flow cytometry buffer) and centrifuged at 4°C, 400 *g* for 5 min. Each antibody was diluted appropriately with the flow cytometry buffer and then 25 μl was added to each cell preparation. The cells were stained on ice for 20 min, with mixing every 5 min. If the used antibodies were conjugated to biotin, the cells, after having been washed twice, were further stained with streptavidinylated fluorochrome. The samples were washed again, and 500–700 μl of flow cytometry buffer were added for analysis. Flow cytometry was performed using a FACSort machine (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). Aggregated cells were excluded from the analysis by gating a forward and a side scatter.

The MoAbs used in this study were as follows: an anti-pan TCR β chain (H57-597) conjugated to biotin and anti-Thy 1·2 (30-H12) conjugated directly to R-phycoerythrin (R-PE) were purchased from PharMingen, FITC-anti-CD4 (YTS191.1.2) and R-PE-anti-CD8α (53.6.7) were purchased from GIBCO BRL (Gaithersburg, MD, USA); streptavidin-R-PE was purchased from GIBCO BRL, anti-αEβ7 (2E7) was presented by Lefrancois *et al.* (22), and FITC-antihamster IgG (H + L) was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Antibodies against mouse L-selectin (MEL-14, rat IgG2a), α4-integrin (R1-2, rat IgG2b), αE-integrin (M290), β7-integrin (FIB27), CD11a (M17/4) and CD3 (145–2C11, hamster IgG) were purchased from PharMingen.

Lymphocyte labelling with carboxyfluorescein diacetate succinimidyl ester (CFSE)

CFDSE (Molecular Probes, Eugene, OR, USA) was dissolved in dimethylsulphoxide to 15·6 mM and a small aliquot (300 μl) was stored in a cuvette sealed with argon gas at −80°C until the experiments were conducted. Lymphocytes (1×10^7) were incubated in CFSE solution (20 μl of stock solution was diluted with 20 ml of RPMI-1640) for 30 min at 37°C. The labelled lymphocytes were centrifuged immediately through a cushion of heat-inactivated fetal bovine serum and washed twice with a cold suspension medium. The cells were resuspended in 0·2 ml of the medium and used within 30 min.

Intravital observation of lymphocyte migration in intestinal mucosa

The intestinal villi were observed from the mucosal surface and lymphocyte migration was also observed. After an intraperitoneal injection of pentobarbital sodium (50 mg/kg), the abdomen was opened via a midline incision. A 7-cm ileal segment ending at the caecal valve was gently extended onto a plate and a longitudinal incision of about 2 cm was made in the middle of the segment by microcautery along its antimesenteric border. The intestine was kept warm and moist by continuous superfusion with physiological saline warmed to 37°C. The adjacent intestinal segment and mesentery were covered with absorbent cotton soaked with Krebs–Ringer solution.

Suitable areas of villus tips were observed from the mucosal surface by an inverted fluorescence microscope (Diaphot TMD-2S, Nikon, Tokyo, Japan) and the observation was recorded by using a videotape recording system. The same area of ileal mucosa was always examined throughout the observation period. The behaviour of fluorescently labelled lymphocytes was visualized on a television monitor by using a fluorescence microscope equipped with a silicon intensified target image tube (SIT) camera with a contrast-enhancing unit (C-2400–08, Hamamatsu Photonics Co., Shizuoka, Japan) according to a method described previously [16,18]. In this setting, the tip of each villus was observed as an oblique circle, and archade microvessels in the villi were also observed. In another set of experiments, a 5-cm ileal segment ending at the caecal valve was chosen for observation of Peyer's patches. Two small incisions in the bowel wall were made and the luminal pressure of the gut loop was maintained at 10 cmH_2O with physiological saline. The microcirculation in Peyer's patches was observed through the serosa by microscope. Epi-illumination was achieved by using filters for excitation at 470–490 nm and for emission at 520 nm. Lymphocytes (1×10^7 dissolved in 1 ml) were injected into a jugular vein of the recipient mice for 3 min. The cell kinetics of the infused lymphocytes, their interaction with microvascular beds and their accumulation in the villus mucosa or Peyer's patches were monitored and recorded continuously on S-VHS videotapes for the first 20 min and then, at 10-min intervals, for 40 min. Lymphocytes adhering to the microvessels of the villus mucosa or Peyer's patches and remaining in the same position without movement for more than 30 s were defined as 'sticking' lymphocytes. The number of sticking lymphocytes was determined in a 1-mm^2 area observed in a video image.