④ ミクロスポーラ症

持続性の下痢を示すが，**米国における消化管の日和見感染症の原因として最も多い**とされている．ミクロスポーラとは類縁の複数種原虫の総称であり，イヌなどのペット動物，魚類，昆虫が保有しているほか，空気中や水中からも検出可能である．

⑤ トキソプラズマ症

細胞内寄生原虫であるトキソプラズマはネコの寄生虫であるが，肉食を通じて感染の機会が多く，米国政府は約40％が原虫陽性と推定している．したがって，米国人や米国在住の邦人ではトキソプラズマ感染を念頭におく必要がある．年間の新感染者は1,000人当たり2～6人と推定され，先天性トキソプラズマ症の頻度も日本と比べて高い．

⑥ イヌ/ネコ回虫症

イヌやネコの回虫による幼虫移行症で網膜下肉芽腫が問題となる寄生虫である．年間7～800例が確認されていて，国民の抗体陽性率は4～7％で，11歳以下の子供に多い．都市部以外の比較的低所得者層で頻度が高い．

⑦ その他

日本で見られる寄生虫症の状況とよく似ている．エキノコックス症は中西部で散見され，旋毛虫症も毎年数十例報告がある．また，**カリフォルニアやフロリダではマラリア感染が復活してきており，今後の監視が必要である．**

Ⅱ ヨーロッパでかかる寄生虫病

ヨーロッパも西欧と東欧，北欧と南欧など自然環境，社会環境が大きく違う．ヨーロッパへの渡航邦人やヨーロッパ出身者の寄生虫感染として特別のものは多くないが，HIV/AIDSに伴う寄生虫相の変化や東欧での政治的混乱の結果，寄生虫感染も増加したことなどが伝えられている．代表的なものを**表1**に示したが，最近の動向も北米より複雑である．

① トキソプラズマ症

北米と同様に食肉生産が盛んでトキソプラズマ感染が多い．ヨーロッパ圏内でも地域差は存在するが，パリでは局所的に住民の80％以上に原虫保有が確認される地域が存在する．日和見感染症として重要である．

② リーシュマニア症

南西ヨーロッパはリーシュマニア症の流行地である．サシチョウバエが媒介する原虫症で，イベリア半島を中心にフランス，イタリアにも存在する．最近ではHIVとの重複感染が新たな流行様態として注目されている[4]．

③ 広節裂頭条虫症

非加熱調理の鮭鱒類を摂食して，腸管内に巨大なサナダムシの寄生をみる．原因食品の市場流通域から北欧に多い．日本近海の日本海裂頭条虫とは別種である．北欧のサナダムシは時に悪性貧血を合併する．

④ 腸管寄生線虫症

ヨーロッパ各国での腸管寄生線虫の問題は在留邦人にとって最近まで問題ではなかった[5]．しかし，**東欧圏の一部では，衛生インフラが崩壊して，回虫，鉤虫などの腸管寄生線虫感染が再興しているとの警告もある．**

⑤ クリプトスポリジウム症

ヨーロッパのAIDS患者の *C. parvum* 感染率は6.7％と報告されている．湖沼，河川以外に，水道水からも原虫が検出されており，「ヨーロッパの水道水は硬水だから下痢する」という日本人旅行者の一部が本症によるものであった可能性は否定できない．

⑥ その他

ロシアや南欧でマラリアの再興がみられ，モスクワでも蚊から感染した症例が発生している．欧州のバベシア症は *B. bovis* によるものもあり，予後は悪い．肉胞子虫は中部ヨーロッパの牛に感染がみられ，免疫低下宿主には病原性を示す．

Ⅲ 予防に必要な注意

北米，ヨーロッパで寄生虫感染に対する注意としては，日本国内での場合とほとんど変わることはない．一方で，北米やヨーロッパとはいえ，南部は亜熱帯性の気候条件であり，マラリアやリーシュマニア症などの熱帯寄生虫症が発生している．とくにシベリアを含むロシアでは都市部でもマラリア流行地になりつつある．また，欧米に流入する途上国から人たちが供血者になった結果，**輸血によるシャーガス病（アメリカトリパノソーマ症）やマラリアなどの感染事例が発生している**（**表2**）[6]．そのような社会背景も北米，ヨーロッパの特殊事情と考えてよいであろう．輸血歴は把握しておくべきである．

表2 アメリカ大陸における輸血用血液のアメリカパノソーマ原虫の混入

場所（国名）	検査数	陽性率
Los Angeles（米国）	998	0.1～1.1％
Cordoba（アルゼンチン）	283,962	7.6％
Sao Paulo（ブラジル）	56,902	2.9％
Santiago（チリ）	2,062	1.5％
Bogoda（コロンビア）	1,012	2.8％

（文献6）より改変）

寄生虫病は北米，ヨーロッパには存在しないのではなく，情報が乏しいに過ぎない，との認識を持ち，AIDSなどを背景にした日和見寄生虫感染症は十分に蔓延していることを渡航者と臨床医の双方が理解しておくことが予防の上で大切である．

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住血吸虫症

——揚子江流域の流行になにが起こりつつあるのか

Schistosomiasis——What is happening along the Yangtze River

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揚子江の歴史と日本住血吸虫症

中国における住血吸虫症は悠久な揚子江の流れとともにある．人類文明が勃興した地域のひとつであるが，おそらくはこの地域への人類の定着とともに住血吸虫症も起こったのであろう．中国国内の住血吸虫症流行地は揚子江中～下流域の低湿地に広がる湖沼型と四川省や雲南省にみられる山丘型の2型に分けられるが，揚子江流域には湖沼型流行地が湖南省，湖北省，江西省，安徽省，江蘇省など広範囲にわたって点在している[1]．この地域に古来，住血吸虫症が存在した確実な証拠は湖南省長沙市にある馬王堆遺跡から出土した女性の遺体から得られた．紀元前200年前後と推定されるその遺体はまるで前日に亡くなったといってよいほどの保存状態で出土し，病理解剖で心筋梗塞が死因であることも判明している．そして，その女性の直腸粘膜から日本住血吸虫卵が確認されたのである．

「三国誌」のハイライトのひとつ，“赤壁の戦い”は西暦208年の出来事で，湖北省と湖南省の境の揚子江の一角がその古戦場跡である．曹操率いる魏の水軍が大敗を喫したことで知られるが，そこはまさに住血吸虫症の濃厚流行地として知られた場所である．史家の多くは，住血吸虫症とは無縁の北方出身の魏の兵士が揚子江に接して急性住血吸虫症を病んだために戦力が大幅に低下したことが敗因のひとつと論じている．近年に眼を向けると毛沢東と住血吸虫症の戦いも歴史的な逸話になりつつある．毛沢東は湖南省韶山の出身で，青年時代は流行地である長沙市内の師範学校で学んでいる．その影響か住血吸虫症には関心が高かったらしく，革命後はただちに住血吸虫症制圧を党の重要政策に据えた．上海近郊の青浦にある住血吸虫記念館の資料によれば，この病気で全村壊滅の悲劇があったことは事実のようである．恐怖の病気であった住血吸虫症に対する彼の想いが「送瘟神」という詩に謳われている．毛沢東はそのなかで古来人民を苦しめた住血吸虫症がいまや制圧に至ったことを高らかに讃えているが，実際には彼の存命中に揚子江流域で，制圧はさほど成功していなかったのである．

著者は1988年以来，揚子江流域，とくに湖南省の住血吸虫症対策にかかわってきた．21世紀に入った現在，揚子江の変わらぬ流れのなかで，そこに住む人びとと住血吸虫の移ろいの行く末を展望してみるのが本稿の目的である．

困難を極める日本住血吸虫症の制圧

中国に存在する住血吸虫症は日本住血吸虫によるものである．いわゆるintestinal schistosomiasisがその病型であり，急性期に消化器症状，慢性期には肝線維症などが流行地住民にみられた．末期症状としては肝硬変も併発して死に至る(図1)．住血吸虫症は水中で幼虫が皮膚から侵入することで感染が成立する．幼虫は淡水産の貝のなかで発育するので，疾病制圧において取られる戦略は終宿主の治療，中間宿主貝の殺滅，および生活用水/糞便の衛生管理である．最近では“終宿主の治療”が住血吸虫症対策の柱となっているが，一方で中

図 1　肝硬変を併発した日本住血吸虫感染者

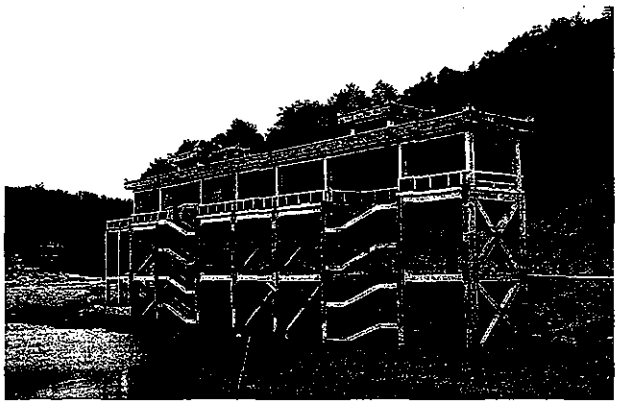

図 2　揚子江流域洞庭湖の観光地
君山の船着き場は渇水期には使用できない

間宿主対策は殺貝剤を散布することで環境汚染の問題がつねに附随してくるため，最近では対策戦略の中心たりえない．

揚子江流域の住血吸虫症対策を困難なものにしているおもな要因は 2 つある．ひとつはヒト以外の動物が終宿主として流行の維持に大きな貢献をしていることである[2]．揚子江流域では水牛が農耕を担う重要な家畜であるが，水牛は日本住血吸虫に対する感染感受性が高い．すなわち，日本住血吸虫症は人獣共通感染症であり，水牛のほかにブタ，ウシ，ヒツジなども日本住血吸虫の終宿主になっている．したがって，終宿主の治療ということはヒトの治療だけでは不十分で，水牛などの保虫宿主も同時に治療しないとヒトの感染を遮断する効果は十分に得られないことになる．しかし，実際には水牛など放牧された家畜一頭一頭の診断と治療を正確に実施することは不可能である．ちなみに，同じ日本住血吸虫症流行地でもフィリピンの水牛はけっして重要な保虫宿主ではない．生物学的に説明を試みているが，中国とフィリピンとで水牛の住血吸虫感染感受性が違うことの機序はいぜんとして不明である．

第 2 の問題は揚子江の自然条件である．揚子江は乾季と雨季とで水位が大きく変化する．その水位差はゆうに 10 m を超え，流域の景観は季節によってまったく異なるものとなる(図 2)．そのことは中間宿主貝の生息面積に直接影響する．乾季には揚子江流域には広大な草地帯が出現し，それが中間宿主である *Oncomelania* 属貝の好適繁殖地となっている．季節によって“有病地”が変化するため，行政対応に大きな支障が生じることになる．揚子江の大洪水がもたらす影響も深刻である．洪水によって中間宿主貝が予測をこえる範囲にまで拡散する結果，あらたな有病地が確認されるようになった．

三峡ダムの予想される功罪

中国有数の景勝地である三峡にダムを建設する計画が正式に承認されたのは 1992 年である．その目的は揚子江中流域の治水や大規模電力開発などで，2009 年に完成をめざす中国の巨大国家プロジェクトである．住血吸虫症の流行にいかなる影響が出るというのであろうか．ダム建設による住血吸虫症流行の拡大ということではエジプトのアスワンハイダム建設の事例を想起される向きもあろうが，日本住血吸虫症に与える影響は事情が異なる．アスワンハイダムでは人造湖が出現して，そこに中間宿主貝が大繁殖したことが問題であったのだが，三峡ダムで問題にしているのは実は下流域に及ぼす効果なのである．揚子江が季節によって水位の上下を繰り返していたものが，ダムによって季節を通じて安定した草地帯が流域に出現することになる．すなわち，下流域に広大な貝の棲息地が確保されることを意味するからである．

その影響についてさまざまなシミュレーションがなされている．現状では流行拡大が懸念される地域はダムに比較的近い湖北省，湖南省に限られ，一方で江西省では幸いに影響がないという予測が

ある．また，三峡ダム建設の目的のひとつに治水効果が上げられていたが，年中行事のような揚子江の大水害が治まるという説と，大自然は人間のちっぽけなダム建設くらいでは変化することはありえない，という説が交わされており，われわれは当然のことながら，その答えを知らない．しかし，確実にいえるのはダム建設によって中国の詩人達に謳われた天下の景観が失われることであり，そのことこそが最大の影響であるのかもしれない．

世界銀行融資と揚子江流域流行地の住血吸虫症対策

中国の日本住血吸虫症流行は，一時的とはいえ，過去10年間に流行の制圧に向けてたしかに前進がみられた．前述のとおり中国政府は1992年から世界銀行の融資を受けて日本住血吸虫症対策の一大国家プロジェクトに取り組んだ．そして，1999年の統計では中国国内の流行地は409郡を数えるが，うち58.2%で流行がほぼ終息したと判定している．流行状況は“流行継続”，“流行制圧”そして“流行駆逐”の3つに区分されるが，“流行継続”から“流行制圧”に改善するだけでも行政的には大きな負担軽減が見込まれる．流行地ではWHOが推奨する住血吸虫症対策法がとられ実効が上がったのである．世界銀行経由の潤沢な予算も相まって当時取りうる最善のアプローチであった[3]．住民の検査と治療はすべて無料であった．そのために住民検診は高いコンプライアンスで実施され，当初の目論みどおりの効果が得られる結果となった．さらに，重要なことは検査実施者の給料支払いの事実である．世界銀行の融資は末端の検査実施者にも労働対価の支払いが保証されるシステムを確立することになった．このように対策実施者，被験者ともにハッピーな8年間であった．

世界銀行の融資が終了した2000年をもって中国では状況が変化した．予算不足のために住民検診は被験者自身によるコスト負担が定められ，また特効薬プラジカンテルの処方にも受益者負担の原則が導入されたのである．中国経済の発展に伴って上海や広州などの沿岸部の繁栄が伝えられるが，内陸部との経済格差は想像以上のものがあり，著者自身が調査にあたった湖南省の農村地帯では農家の平均年収が1,000元程度(日本円で15,000円くらい)であった．そこに治療薬の自己負担が導入された結果，プラジカンテルの代金として1回20元を感染陽性者が支払うようになった．けっして軽い負担とはいえない金額である．有料の検査を受けて有料の治療を受けるというシステムであれば，今後ますます受診者が減少することは確実である．

図3　湖南省内にある住血吸虫症防治ステーション
保健所と病院を合わせたような機能をもつ

さらに並行して生じた問題は検査実施者の給料未支給の問題である．揚子江流域の各省は住血吸虫症対策の行政的スキームを構築している．末端には“対策ステーション”が村に設置してあり，日本の保健所を小規模にしたものといえば想像は容易であろう(図3)．著者は2002年の時点で，職員の給料遅配は日常的になっていると聞いた．検査室の実態も機器，消耗品など，厳しい予算カットの結果，実施するためには住民からの料金徴集の徹底が必要で，住民は自発的に受診を希望しないようになっていた．2004年現在，すでに正確な疫学情報が把握できなくなったとして本年秋から第三次の住血吸虫症全国調査の実施を中国政府は決定している．

揚子江流域から住血吸虫症をなくすために

毛沢東が願ってやまなかった揚子江流域からの住血吸虫根絶はいかにして達成できるであろうか．感染症制圧をもっとも効率的に推進できるの

表 1　住血吸虫ワクチン開発に関するカイロ会議（1997 年 5 月）のまとめ

① 研究からワクチン実用化に進む段階に至った
② GST，パラミオシン，MAP-4/TPI，Sm 14 については臨床試験の後，GMP グレードで抗原の大量精製を行うべきである
③ IrV5 と MAP-3/Sm 23 の DNA ワクチン化に向けた研究を進めるべきである
④ 上記の他に有望なワクチン抗原を考慮するべきである

はワクチンである．住血吸虫は治療してもすぐに再感染するように，いわゆる“二度なし”免疫は成立しない．しかし，日本でも流行地で生育した人と非流行地から移入した人とで住血吸虫のかかりやすさや病気の重篤度が違うことがあったように，日本住血吸虫感染にも部分免疫は成立しうると考えられる[4]．そこで，部分的ではあっても宿主の防御免疫を賦活してやれば，集団の感染濃度が低下し，やがてその虫を駆逐できるであろう，というのが住血吸虫をはじめとする蠕虫ワクチンの考え方である．住血吸虫ワクチンとしては WHO や地域ネットワークなどを通じて開発戦略が討議されてきた（「サイドメモ」参照）．これまでに表に示したような抗原がワクチン候補として同定されている（表 1）．

日本住血吸虫症は他の住血吸虫に比べてワクチン開発の好適な標的である．その理由はこの寄生虫病が人獣共通感染症であるため，ヒトでの実用化試験が倫理的に困難であっても水牛など家畜動物に効果が認められれば，疾病制圧に一定の貢献がなされることになるからである．日本住血吸虫ワクチンとしていくつかのものがマウスの実験で効果が確認されており，ブタなどの大形家畜動物を用いた試験に進みつつある[5]．この分野では中国，日本，オーストラリアでワクチン実用化のための共同研究が進められている．パラミオシンは現時点でもっとも期待の大きいワクチン標的抗原であるが，長崎大学・平山らは江西省との共同研究でブタにおける効果を検討している．約 40%の感染防御効果が観察されているが，用いるアジュバントの選択など検討が必要である．オーストラリア QIMR の MacManus らは中国の水牛でパラミオシンによるワクチン効果を検討した．残念ながら有意な防御効果は観察できていないが，水牛の免疫に必要な抗原量などを知る予備試験としては情報を得ている．著者らは湖南省においてカルパインのワクチン効果をブタで検討した．マウスで観察されたような有意な防御効果を観察することはできなかったが，明らかな産卵抑制効果があったことは発病阻止ワクチンとしての期待は残された．いずれにせよ，家畜動物を用いたワクチン研究はコストがかかりすぎる点が問題である．そこで今後の方針としては，ワクチン開発のためのワークショップを通じて候補分子を絞り込み，国際協力体制の下で十分な数の家畜動物を用いて試験していくことの合意が得られている．近い将来は中国における住血吸虫症対策としてヒトを治療し，家畜動物にワクチンを用いることで，住血吸虫症根絶をめざしたいと考えている．

サイドメモ

住血吸虫ワクチン

蠕虫ワクチンの意義は他のワクチン開発戦略と根本的なところで異なっている．細菌，ウイルス，原虫に対するワクチンは，sterile immunity，すなわち 1 個体たりとも侵入を許さないことが要求される．それは細菌や原虫は宿主体内で増殖するからであるが，蠕虫は宿主体内で例外を除いては増殖することができない．その結果，蠕虫ワクチンでは部分的な感染防御が誘導できれば，宿主の寄生個体数を減少させる効果が期待されることになる．WHO では住血吸虫ワクチンに対して“40%の感染防御効果”を開発の必要条件として設定した．表 1 はそれをクリアしたワクチン候補であるが，それ以外にも候補分子が同定されつつある．住血吸虫排除の機序は十分にはわかっていないため，どのような免疫エフェクターを導いたらよいか，議論があるところであるが，通常の蛋白ワクチンに加えて，DNA ワクチンも各種実験動物で試みられている．

おわりに

揚子江流域を含むアジアの日本住血吸虫症対策は感染率を軽減することには成功したが，これをさらにゼロにするための事業展開がはかられている．しかし，中国においても住血吸虫症は“neglected

diseases"に位置づけられる傾向にあったことは今後の本症再興の可能性を示唆するものである．一般的にいって中国の住血吸虫症対策はしだいに手薄になりつつあるように思われる．悠久の歴史のなかで揚子江の変わらぬ流れを考えるとき，広大な大地から住血吸虫症をなくすことは不可能なのかもしれないという気になる．近未来の歴史のなかで，たとえば三峡ダム運用を機に住血吸虫流行が拡大したという記述が加わることのないように願っているが，敵はけっして容易な寄生虫でないことは確かである．

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Immunological characteristics of patients infected with common intestinal helminths: results of a study based on reverse-transcriptase PCR

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To determine whether common helminth infections could modify the intestinal immunopathological status of the host, the expression in the human duodenal mucosa of cytokines, eosinophil- and mast-cell-specific molecules and monosaccharide transporters of the glucose-transporter (GLUT) family was explored. The 31 subjects were all patients at the gastro-intestinal disease unit of Nongkhai Hospital, Thailand. Four of the 10 patients who presented with eosinophilia (≥6.0% of their leucocytes were eosinophils), and five of the other 21 patients, had intestinal infections with helminths when they presented or within the previous 3 months. Studies based on semi-quantitative, reverse-transcriptase PCR revealed that the interleukin-5/interferon-γ ratio was significantly higher in the non-eosinophilic, helminth-infected patients than in the non-eosinophilic, uninfected patients, whereas the IgE receptor type I (FcεRI)/mast-cell tryptase ratio was significantly higher in the eosinophilic, helminth-infected patients than in the eosinophilic, uninfected patients. Expression of Charcot–Leyden-crystal protein, GLUT-1 and GLUT-5, however, showed no significant inter-group differences. Principal-components analysis of the data on eosinophils, interleukin-5, interferon-γ, FcεRI and mast-cell tryptase revealed that one principal component could discriminate the patients who had helminth infection from the non-eosinophilic, uninfected patients, but not from the eosinophilic, uninfected patients. These results indicate that, whatever the intestinal pathology, patients infected with common intestinal helminths tend to develop a mucosal immunological response of the Th2 type.

High prevalences of infection with intestinal helminths were revealed when, in 2000 and 2002, stool samples from humans living in Nongkhai province in north–eastern Thailand were examined. Data collected by the provincial health office indicated that, of the general public (and schoolchildren) checked in each of the two study years, 22.0%–33.3% (19.3%–28.2%) were infected, and 5.1%–7.2% (3.5%–11.4%) and 3.8%–9.0% (0.8%–2.6%) were excreting hookworm and *Opisthorchis* eggs, respectively (unpubl. obs.). *Ascaris lumbricoides*, *Strongyloides stercoralis* and *Taenia* accounted for most of the other infections detected (unpubl. obs.). The rather high variation seen between the results in 2000 and those recorded in 2002 may reflect differences in the local communities examined in each year, and/or temporal differences in anthelminthic use. The traditional agricultural and food-handling practices of the region probably account for most of the infections with food-borne or soil-transmitted helminths. Although infection with common intestinal helminths does not necessarily cause overt clinical signs, long-term infections may induce malnutrition and result in retardation

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of the intellectual and physical growth of children (Oberhelman *et al.*, 1998).

Intestinal infections with helminths such as *Ascaris* and hookworm induce expansion of the Th2 lymphocyte subset that regulates IgE antibody production, eosinophilia and mastocytosis (Pritchard *et al.*, 1995; Cooper *et al.*, 2001). Responses of the Th2 type may play an important role in protection against helminth infections, and may counterbalance any potentially damaging Th1 responses (Pritchard and Brown, 2001). Helminth infections may, however, impair immune responses to viruses, bacteria or protozoa, and even those to oral vaccines, through the antagonistic effects of Th2 cytokines on the expansion of the Th1 lymphocyte subset (Cooper *et al.*, 2001). In rodents, infection with intestinal nematodes such as *Nippostrongylus brasiliensis* and *Trichinella spiralis* not only causes the induction of Th2 cytokines, such as interleukin-4 (IL-4), IL-5 and IL-13, but also induces IgE antibody responses, eosinophilia and intestinal mastocytosis (Matsuda *et al.*, 1995; Garside *et al.*, 2000). In such experimental nematode infections, various pathological changes occur in the intestinal mucosa of the hosts, including partial villus atrophy and crypt hyperplasia, together with decreases in the activities of the brush-border enzymes sucrase and alkaline phosphatase, alterations in the expression of hexose, amino-acid and peptide transporters, and deterioration in epithelial permeability and barrier function (Perdue *et al.*, 1989; Hyoh *et al.*, 1999, 2002; Sekikawa *et al.*, 2003). In human intestinal parasite infections, however, it remains unclear whether Th2-associated responses dominate in the intestinal mucosa of the host. In the present study, in order to determine the immunopathological status of the infected human intestinal mucosa, the expression of the cytokines interferon-γ (IFN-γ), IL-5 and IL-13, as well as marker molecules of mast cells and eosinophils in the duodenal mucosa, was investigated in patients from Nongkhai, where intestinal helminth infection is clearly not uncommon. Two members of the glucose-transporter (GLUT) family, GLUT-1 and GLUT-5, were also investigated, to evaluate the functional status of the absorptive cells. GLUT-5 is located in the brush border of intestinal absorptive cells and has an important role in the absorption of fructose from the intestinal lumen (Gould and Holman, 1993). GLUT-1, on the other hand, is expressed in almost every tissue, and its expression has been reported to have a strong association with cellular glycolysis, which is accelerated, with the up-regulation of GLUT-1, after various cellular stresses, including hypoxia (Wertheimer *et al.*, 1991)

PATIENTS AND METHODS

Patients

Patients who visited the gastro-intestinal disease unit at Nongkhai Hospital, because of symptoms such as abdominal discomfort, abdominal pain and vomiting (of various severities and durations) were enrolled in the study if they (1) did not have a dysenteric disease; (2) required fibre-optic examinations of their upper gastro-intestinal tract, for the diagnostic evaluation of their diseases; and (3) gave their written, informed consent for the collection, at the time of their diagnostic endoscopies, of duodenal mucosal biopsies (for the investigation of cytokine and inflammatory-mediator expression in the duodenal mucosa). Overall, 31 patients met the inclusion criteria and were enrolled. A stool sample was collected from each subject, in the week prior to the fibre-optic examination, and used to make thick and thin smears that were checked for protozoa and the ova and larvae of helminths.

Tissue Processing

A piece of the duodenal mucosa 3 cm distal to Vater's papilla was obtained from each patient. Each of these biopsies was immediately immersed in 1 ml of RNA-preservation solution (RNAlater®; Ambion, Austin, TX)

and kept in a refrigerator for 4–50 days (mean = 22.6 days) until the RNA could be extracted. Total RNA was extracted using TRIzol® reagent (Life Technologies, Rockville, MD), and 5-μg aliquots of RNA were then reverse-transcribed, at 42°C for 50 min, in 20 μl reverse-transcription buffer containing 5 mM $MgCl_2$, 1 mM deoxynucleotide-triphosphate (dNTP) mixture, 1 U RNase inhibitor/ml, 250 U AMV (avian myeloblastosis virus) reverse transcriptase/ml, and 0.125 mM oligo dT primer (Takara RNA LA PCR kit; Takara Biomedicals, Osaka, Japan).

PCR

For the PCR, 1-μl aliquots of synthesised complimentary DNA were added to PCR buffer containing 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.025 U LA Taq DNA polymerase (Takara RNA LA PCR kit)/ml, and 0.2 mM of each sense and antisense primer (see Table 1) in a final volume of 25 μl. The thermocycler was set to give cycles of 30 s at 94°C, 30 s at 62°C and 30 s at 72°C. To determine the optimal numbers of PCR cycles, the densities of the bands produced, from one representative sample, when the products from different numbers of PCR cycles were subjected to electrophoresis, were compared. This allowed the number of PCR cycles that allowed the best comparison of the levels of gene expression to be determined, for each molecule of interest.

DENSITY ANALYSES OF PCR PRODUCTS

To separate the products of each PCR, 8-μl aliquots of the amplified products were subjected to electrophoresis on agar, stained with ethidium bromide, and visualized with ultra-violet trans-illumination. The banding patterns were recorded using a charge-coupled-device (CCD) camera (ATTO, Tokyo, Japan), and the density of each band was evaluated using NIH Image software (National Institutes of Health, Bethesda, MD). The band densities were normalized relative to those of β-actin.

RESULTS

The baseline characteristics of the 31 patients enrolled in the study are summarized in

TABLE 1. *The primer pairs used for the PCR amplifications*

	Primers	Size of expected product (bp)
Interferon-γ	5'-GGGTTCTCTTGGCTGTTACTG-3' 5'-GACAGTTCAGCCATCACTTGGA-3'	384
Interleukin-5	5'-GAAATTCCCACAAGTGCATTGG-3' 5'-CTTTCTATTATCCACTCGGTGTTC-3'	335
Interleukin-13	5'-AGGAGCTGGTCAACATCACC-3' 5'-GTTGAACCGTCCCTCGCGAA-3'	296
IgE receptor type I (FcεRI, α chain)	5'-TCAGTGACTGGCTGCTCCTT-3' 5'-GGGGTTTGGCTTAGGATGTG-3'	437
Mast-cell tryptase β1 (McTr)	5'-TCAGCAGGATCATCGTGCAC-3' 5'-TGGGGACATAGTGGTGGATC-3'	507
Charcot–Leyden-crystal protein	5'-TACCCGTGCCATACACAGAG-3' 5'-CTCTCCACACTTGCACCATC-3'	375
Glucose transporter 1 (GLUT-1)	5'-ATCGTCAACACGGCCTTCAC-3' 5'-AAGCCGGAAGCGATCTCATC-3'	458
Glucose transporter 5 (GLUT-5)	5'-GGTACAACGTGGCTGCTGTC-3' 5'-CATGGGGACCACGTTGGAAG-3'	347
β-Actin	5'-TCAGAAGGATTCCTATGTGGGC-3' 5'-CCATCACGATGCCAGTGGTA-3'	317

TABLE 2. *The baseline demographic and laboratory data for the 31 subjects*

	Total	Helminth-infected	Uninfected	With peptic ulcer	Without ulcer
No. of subjects	31	9*	22	6†	25
No. of males/females	22/9	9/0	13/9	5/1	17/8
MEAN VALUE, (S.D.) AND [no. of subjects for whom data were available]					
Age (years)	45.8 (13.5) [31]	43.3 (10.8) [9]	46.8 (14.5) [22]	47.8 (8.9) [6]	45.3 (14.6) [25]
Albumin (g/dl)	3.9 (0.7) [23]	4.1 (0.6) [8]	3.8 (0.7) [15]	3.5 (1.0) [5]	4.0 (0.6) [18]
Haemoglobin (g/dl)	13.3 (2.8) [29]	13.5 (2.2) [8]	13.0 (3.0) [21]	12.3 (4.1) [6]	13.4 (2.4) [23]
Eosinophils (% of leucocytes)	4.8 (4.6) [31]	5.3 (4.6) [9]	4.6 (4.7) [22]	3.1 (3.2) [6]	5.2 (4.9) [25]

*With intestinal helminth infection (two with *Strongyloides*, six with *Taenia* and one with *Ascaris*) on presentation or within previous 3 months.
†One with ulcer in the duodenum and five with stomach ulcers.

Table 2. All the patients investigated were from Nongkhai province, where agriculture is the main industry. The oesophago-gastro-duodenal fibre-optic examinations carried out for the diagnostic evaluations revealed 17 cases of chronic gastritis, six of peptic ulcer, four of oesophagitis, and three of erosive gastritis; the other three cases showed no particular findings. The stool examinations revealed two cases excreting *Strongyloides stercoralis* larvae and two others excreting the ova and/or proglottids of *Taenia*. The intensities of both *S. stercoralis* infections detected were low, with only one or two rhabditoid larvae detected in each thin faecal smear, and neither patient found excreting the larvae had eosinophilia (0.5% and 2.1% of their leucocytes were eosinophils). The intensity of *Taenia* infection could not be determined but it is known that infection with *Taenia* usually consists of a single worm (Beaver *et al.*, 1984). The medical records of the patients who were found stool-negative for helminths revealed that five had had intestinal infections with helminths in the 3 months before their presentation: one had had an *Ascaris* infection (scored ++ for the number of *Ascaris* ova seen on a thin faecal smear), and another four had each had a *Taenia* infection (with ova or proglottids in their stools). Overall, therefore, nine of the patients investigated were considered 'infected', having being found positive for intestinal helminths either at their enrolment or in the previous 3 months.

None of the 31 patients appeared to have an enteric infection with parasitic protozoa such as *Entamoeba histolytica* or *Giardia lamblia*, either at enrolment or whenever checked in the previous 3 months. The results of urea tests for *Helicobacter pylori* infection, carried out on all 31 subjects at enrolment, were positive for seven patients, all of whom appeared smear-negative for helminths at enrolment and had no obvious history of helminth infection in the previous 3 months. Only one of the 31 subjects (an asthmatic with mild dyspnea, as well as *S. stercoralis* infection) had a history or the signs and symptoms of an allergic disease. In terms of age, gender and the results of the laboratory tests, the helminth-infected subjects were similar to the uninfected, and the patients with peptic ulcers were similar to those without such ulcers (Table 2).

To determine the mucosal immunological status of each subject, the levels of expression of IL-13, IL-5, IFN-γ, Charcot-Leyden-crystal protein (CLC), IgE receptor type I (FcεRI), mast-cell tryptase (McTr), GLUT-1 and GLUT-5 in the duodenal mucosa were evaluated by reverse-transcriptase PCR (RT-PCR). Expression of the Th2 cytokines IL-5 and IL-13 was detectable in the biopsies from 20 (64.5%) and eight (25.8%) of the patients, respectively, whereas expression of the Th1 cytokine IFN-γ and of all the other molecules investigated was detectable in all of the biopsies examined (Fig. 1). Expression

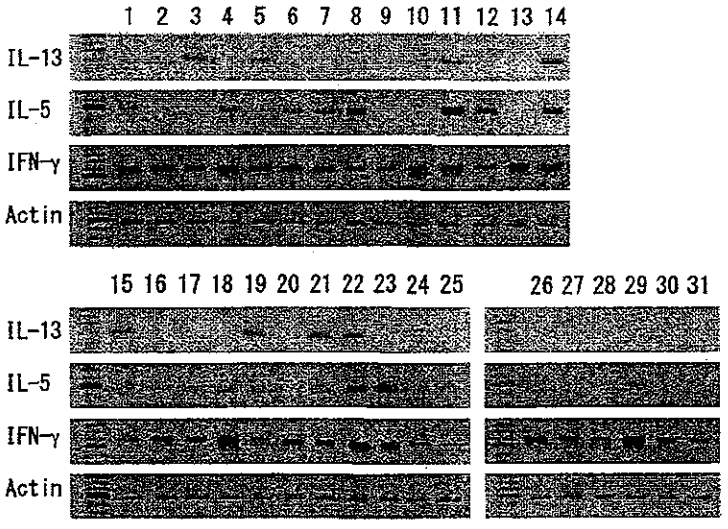

FIG. 1. Some of the PCR results, showing the expression of interleukin-13 (IL-13), interleukin-5 (IL-5), interferon-γ (IFN-γ) and, for reference, β-actin (Actin) in the duodenal mucosa of the 31 patients. Samples from patients with helminth infection on presentation or in the previous 3 months were run in lanes 15–23.

of IL-5 showed no relationship with subject age, the period of sample preservation, the gender of the subject, the presence of a peptic ulcer, or helminth infection (Fig. 2). This cytokine was, however, expressed by a significantly higher proportion of the subjects with peripheral-blood eosinophilia (defined here as those in whom at least 6% of leucocytes were eosinophils) than of the other subjects (Fig. 2). The expression of IL-13 did not show significant association with eosinophilia or helminth infection.

For some of the data analysis, the subjects were split into four groups: those with eosinophilia, with and without helminth infection, and those without eosinophilia, with and without helminth infection. Five of the 21 patients who were not eosinophilic and four of the 10 with eosinophilia were considered infected with intestinal helminths. Although the overall levels of IL-5 expression did not differ between the eosinophilic and non-eosinophilic subjects, the levels of IFN-γ expression were significantly higher in the eosinophilic subjects (Table 3). Among the subjects who did not have eosinophilia, the IL-5/IFN-γ ratio, which might reflect one aspect of the Th1/Th2 balance in each individual, was significantly higher in the helminth-infected subjects than in the uninfected; no such difference was observed among the eosinophilic patients (Table 3).

The expression levels of CLC, FcεRI, McTr, GLUT-1 and GLUT-5 showed no significant differences between the subjects groups (Table 3). Although the expression levels of FcεRI and McTr might be directly related to mast-cell density in the mucosa, it has been reported that nematode infection or exposure of mast cells to a large amount of IgE antibodies results in a striking up-regulation of FcεRI expression on mast cells (Chen and Enerback, 1996; Shaikh *et al.*, 1997; Yamaguchi *et al.*, 1997). Among the eosinophilic subjects of the present study, the FcεRI/McTr ratio, which might represent the FcεRI expression level/mast cell in each individual, was found to be significantly higher in the helminth-infected than in the uninfected; no such difference was observed in the patients who were not eosinophilic (Table 3).

To examine whether a component composed of eosinophils, IL-5, IFN-γ, FcεRI

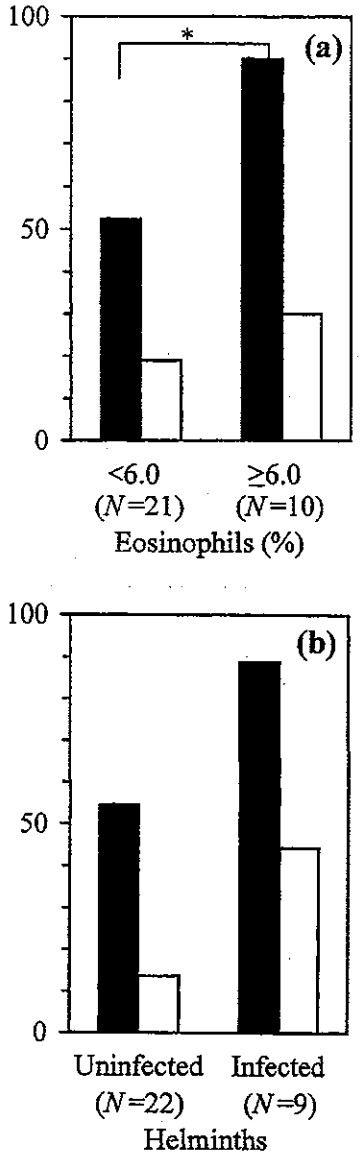

FIG. 2. The frequencies of expression of interleukin-5 (■) and interleukin-13 (□) in the duodenal mucosa — of the 10 patients with eosinophilia and the 21 without (a), and in the nine patients with helminth infection (on presentation or in the previous 3 months) and the 22 without (b). The frequencies of positivity for interleukin-5 expression were significantly higher for the eosinophilics than for the non-eosinophilics ($P=0.041$).

and McTr could be a predictor of helminth-infection status, principal-component analysis was carried out. This is a method to replace the original set of variables (in which one variable might not be independent of another) by one or a few variables (principal components) that account for the bulk of the variation observed, making the results easier to understand. As shown in Table 4, although the principal component identified gave a score for the helminth-infected patients that was significantly different to that for the uninfected patients without eosinophilia, it could not discriminate between the helminth-infected patients and the uninfected patients with eosinophilia.

DISCUSSION

In rodent models, *Trichinella spiralis* infection has been reported to induce a predominantly Th2-type cytokine response in the intestinal lymph (Ramaswamy *et al.*, 1996). In addition, comparative studies of mucosal and peripheral cytokine responses in *Trichuris muris*-infected mice indicated that the dominant cytokine responses of the mesenteric lymph nodes can be detected by sampling peripheral-blood lymphocytes (Taylor *et al.*, 2000). Although intestinal helminth infections such as ascariasis and strongyloidiasis have been reported to induce the production of type-2 cytokines by peripheral-blood mononuclear cells (Cooper *et al.*, 2001; Porto *et al.*, 2001), the effect of helminth infections on the expression of these cytokines in the intestinal mucosa has not been elucidated. Besides cases of strongyloidiasis and ascariasis patients, the present study included six cases of taeniasis. Cases of human taeniasis are not uncommon in Thailand. Most are attributed to *Ta. saginata*, on the bases of the parasite's unarmed scolex and the uterus-branching pattern of the proglottids. None of the subjects of the present study showed any clinical sign of cysticercosis (caused by *Ta. solium*). Recently, it has been suggested that at least some cases of human taeniasis in Thailand are caused by *Taenia saginata asiatica*, a subspecies that is distributed throughout many other Asian countries,

TABLE 3. *The mean (S.E.) levels of expression (as proportions of the level of expression of the β-actin used as a standard), of interleukin-5 (IL-5), interferon-γ (IFN-γ), Charcot–Leyden-crystal protein (CLC), mast-cell tryptase β1 (McTr), IgE receptor type I (FcεRI), glucose transporter 1 (GLUT-1) and glucose transporter 5 (GLUT-5) in the duodenal mucosa, and some mean (S.E.) ratios between them*

	Non-eosinophilics			Eosinophilics		
	Total ($N=21$)	Uninfected ($N=16$)	Infected ($N=5$)*	Total ($N=10$)	Uninfected ($N=6$)	Infected ($N=4$)†
IL-5	0.21 (0.09)	0.14 (0.08)	0.45 (0.33)	0.36 (0.10)	0.33 (0.11)	0.39 (0.24)
IFN-γ	0.65 (0.11)	0.66 (0.14)	0.62 (0.17)	1.20 (0.22)‡	1.32 (0.28)‡	1.02 (0.42)
IL-5/IFN-γ	0.27 (0.09)	0.15 (0.06)	0.65 (0.29)§	0.41 (0.17)	0.39 (0.24)	0.45 (0.25)
CLC	0.69 (0.10)	0.66 (0.11)	0.78 (0.31)	0.74 (0.19)	0.92 (0.27)	0.48 (0.21)
FcεRI	0.52 (0.11)	0.42 (0.09)	0.82 (0.32)	0.58 (0.09)	0.56 (0.12)	0.60 (0.14)
McTr	2.16 (0.19)	1.98 (0.18)	2.76 (0.52)	2.79 (0.44)	3.41 (0.57)	1.87 (0.13)
FcεRI/McTr	0.21 (0.04)	0.20 (0.04)	0.26 (0.06)	0.23 (0.05)	0.16 (0.03)	0.33 (0.09)¶
GLUT-1	1.17 (0.15)	1.13 (0.20)	1.23 (0.17)	0.90 (0.23)	1.01 (0.32)	0.74 (0.32)
GLUT-5	1.17 (0.09)	1.12 (0.10)	1.32 (0.25)	1.25 (0.13)	1.41 (0.17)	1.00 (0.12)

*Two with *Strongyloides* and three with *Taenia*, on presentation or within previous 3 months.
†Three with *Taenia* and one with *Ascaris*, on presentation or within previous 3 months.
‡Significantly higher than the corresponding values for the non-eosinophilics ($P<0.05$).
§Significantly higher than the value for the uninfected non-eosinophilics ($P<0.05$).
¶Significantly higher than the value for the uninfected eosinophilics ($P<0.05$).

TABLE 4. *The results of the principal-components analysis*

Parameter	Value
Eigenvalue (and percentage of variation explained)	2.13966 (42.8)
UNROTATED FACTOR LOADINGS	
Eosinophils	−0.55202
Interleukin-5	−0.43733
Interferon-γ	−0.72827
IgE receptor type I (FcεRI)	−0.61319
Mast-cell tryptase β1 (McTr)	−0.85866
MEAN (S.E.) COMPONENT SCORES	
Others (uninfected patients without eosinophilia; $N=16$)	0.761 (0.283)
Helminth-infected* ($N=9$)	−0.327 (0.375)†
Eosinophilia (uninfected patients with eosinophilia; $N=6$)	−1.538 (0.733)‡

*With intestinal helminth infection on presentation or within previous 3 months.
†Significantly different to other component scores ($P<0.05$).
‡Significantly different to other component scores ($P<0.01$).

including Taiwan, Korea, Indonesia and the Philippines (Fan *et al.*, 1990; Eom and Rim, 2001). Compared with those on intestinal nematode infections such as ascariasis, hookworm disease and strongyloidiasis, there have been relatively few studies to determine whether intestinal tapeworms could induce Th2-type responses. In murine models, however, a predominantly Th2 response has been reported to occur during the lumenal phase of both *Hymenolepis nana* and *H. diminuta* infection (Conchedda *et al.*, 1997; Palmas *et al.*, 1997). Experimental infection of gerbils or hamsters with adult *Ta. solium* induced goblet-cell hyperplasia and mastocytosis, indicating that Th2-related responses occurred in the intestine (Avila *et al.*, 2002). Peripheral-blood eosinophilia was induced

by the experimental infection of human volunteers with *Ta. saginata* or *Ta. saginata asiatica* (Chao *et al.*, 1988; Tesfa-Yohannes, 1990). Thus, it seems likely that intestinal infection with *Taenia* shares similar immunological features with intestinal infection with other common helminths.

In the present study, the Th2 cytokines IL-13 and IL-5 were expressed in the duodenal mucosa of 25.8% and 64.5% of the patients, respectively, whereas the Th1 cytokine IFN-γ was expressed in all of the patients. The biopsied tissues examined in the present study had been preserved in an RNA-preservation fluid, in a refrigerator, for varying lengths of time (4–50 days), before total RNA was extracted. No correlation was observed, however, between the frequencies or intensities of IL-13, IL-5 or IFN-γ expression and the duration of the tissue preservation, indicating that the frequencies and intensities observed reflected the immunological status in the mucosa. Although IL-5 is an important factor in eosinophil differentiation, proliferation, survival and migration, healthy individuals appear not to express IL-5 in the duodenal mucosa (Wallaert *et al.*, 1995; Vandezande *et al.*, 1999), indicating that there are few, if any, Th2-cytokine-producing cells in the non-lymphoid tissues of healthy individuals. In the present study, IL-5 expression in the duodenal mucosa was associated with peripheral-blood eosinophilia but CLC expression did not show a clear association with either IL-5 expression or peripheral-blood eosinophilia. Thus, locally produced, chemotactic or chemoattractant factors might play more important roles than IL-5 in the mucosal infiltration of eosinophils.

Patients with peripheral-blood eosinophilia showed significantly higher levels of IFN-γ expression in the duodenum than patients with lower numbers of eosinophils, indicating that Th1 and Th2 responses might occur concomitantly in the duodenal mucosa of these patients. Alternatively, since IFN-γ is produced not only by $CD4^+$ cells but also by $CD8^+$ and NK cells, the enhanced IFN-γ expression might reflect the abundance of cytotoxic cells in the mucosa. The IL-5/IFN-γ ratio is considered to reflect, at least in part, the Th1/Th2 balance. In the present study, the helminth-infected non-eosinophilic patients showed significantly higher IL-5/IFN-γ ratios than the uninfected non-eosinophilic patients, although the effect of helminth infection was not clear in patients with peripheral eosinophilia. It seems that, in the helminth-infected patients with peripheral eosinophilia, a more mixed Th1/Th2 status had developed rather than a shift to a simple, Th2-dominant status. Interestingly, however, the FcεRI/McTr ratios in the eosinophilic patients were significantly higher in the helminth-infected than in the uninfected. Since nematode infection or exposure of mast cells to a large amount of IgE antibodies are reported to result in striking up-regulation of FcεRI expression on mast cells (Chen and Enerback, 1996; Shaikh *et al.*, 1997; Yamaguchi *et al.*, 1997), the FcεRI/McTr ratio might reflect, at least in part, the functional status of the mast cells. Thus, in patients with eosinophilia, helminth infection seems to exert a unique Th2-related effect on the mucosal immunology. Principal-components analysis of variables including eosinophils, IL-5, IFN-γ, FcεRI and McTr could identify a component that reflects one aspect of Th2-type immune responses. This component could discriminate helminth-infected patients from the non-eosinophilic, uninfected patients, but not from the uninfected patients who had eosinophilia. All the uninfected patients with eosinophilia were clinically diagnosed as having non-ulcer dyspepsia. At least some of these patients might have had eosinophilic gastro-enteritis with an unknown allergic basis. On the other hand, it is also possible that some of the patients were infected with some kinds of parasites that migrate in the viscera, although none of them showed clinical signs of hepatitis, pneumonitis or cutaneous creeping disease.

Taken together, the present findings indicate that infections with common intestinal helminths, including taeniasis, are likely to

modify mucosal immunology so that it shifts to a Th2-predominant status. In rodents, expression of the apical-surface monosaccharide transporters SGLT-1 and GLUT-5 was recently found to be significantly down-regulated following infection with the intestinal nematode *N. brasiliensis* (Sekikawa *et al.*, 2003). In the present study, however, no decrease of monosaccharide-transporter expression was observed in patients with helminth infection. The impact of intestinal helminths on human nutrition remains to be clarified.

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VILLUS EPITHELIAL INJURY INDUCED BY INFECTION WITH THE NEMATODE *NIPPOSTRONGYLUS BRASILIENSIS* IS ASSOCIATED WITH UPREGULATION OF GRANZYME B

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ABSTRACT: Intestinal parasite infections induce thymus-dependent villus atrophy, but the effector mechanisms directly responsible for the development of villus atrophy are not thoroughly understood. In this study, we analyzed the expression of cytotoxic factors or ligands in athymic nude rnu/rnu rats and their littermate euthymic rnu/+ rats infected with the nematode *Nippostrongylus brasiliensis*. Morphometric analyses showed that partial villus atrophy developed 10 days after infection in euthymic but not in athymic rats, whereas crypt hyperplasia occurred in both types of animal. Reverse transcription–polymerase chain reaction analyses of the isolated jejunal epithelial fraction showed that the development of villus atrophy in euthymic rats was positively correlated with an increase of granzyme B transcript levels but not with Fas ligand or tumor necrosis factor-α expression. In addition, the number of granzyme B–immunoreactive cells was increased significantly in euthymic rat villus epithelium and the propria mucosa after infection. The $CD8^+$ cell number did not change significantly. Collectively, these findings showed that significant increases in the number of cells that express the cytotoxic factor granzyme B occur in the nematode-infected small intestine of immunocompetent hosts. The type of cells that express granzyme B and their role in the progression of enteropathy remain to be elucidated.

Intestinal villus atrophy with crypt hyperplasia has been reported in a number of intestinal diseases, such as celiac disease, giardiasis, intestinal helminth infection, autoimmune enteropathy, graft-versus-host disease, and allograft rejection of transplanted small intestine (MacDonald, 1992). Infection of rodents with the intestinal nematode *Nippostrongylus brasiliensis* induces partial villus atrophy and crypt hyperplasia in the jejunum together with various cytological alterations, such as decreases in the activities of the brush border enzymes sucrase and alkaline phosphatase, and causes deterioration of the epithelial permeability and barrier function (Symons, 1965; Nolla et al., 1985; Lunn et al., 1986; Perdue et al., 1989; Hyoh et al., 1999). The partial villus atrophy develops at the climax of infection, 7–10 days after infection, and disappears after T cell–dependent nematode clearance 12–14 days after infection. These histological alterations have been attributed largely, if not exclusively, to activation of mast cells in the intestinal mucosa accompanied by the release of mediators such as rat mast cell protease II (RMCP II) and the generation of leukotrienes (Woodbury et al., 1984; Perdue et al., 1989; D'Inca et al., 1990). On the other hand, anaphylactic release of RMCP II induces no significant change in mucosal histology (Scudamore et al., 1995), and mast cell–deficient *Ws/Ws* rats develop intense villus atrophy, as do wild-type +/+ rats, after nematode infection, suggesting that mast cell activation is not relevant to the progression of villus atrophy (Hyoh et al., 1999).

Ferguson and Jarrett (1975) reported that thymus-deprived (B) rats did not develop villus atrophy despite the fact that *N. brasiliensis* infection was prolonged. Similarly, in athymic rnu/rnu rats, villus atrophy does not develop until at least 10 days after infection, although it does develop 21 days after infection together with the occurrence of mucosal damage involving impaired barrier and digestive functions (D'Inca et al., 1992; McKay et al., 1995). These results indicate that the development of villus atrophy is thymus dependent, at least in the early period of infection, despite the fact that the innate immunity in athymic rats could induce villus injury and even villus atrophy over time. However, the mechanisms whereby T cells regulate enteropathy or the development of villus atrophy are not thoroughly understood. In this study, we analyzed the gene expression of granzyme B, Fas ligand (FasL), and tumor necrosis factor-α (TNF-α), which are expressed mainly in cytotoxic T lymphocytes (CTL), natural killer (NK) cells, or macrophages, as well as the tissue distribution of granzyme B–immunoreactive cells in athymic rats and their littermate euthymic rats infected with *N. brasiliensis*.

MATERIALS AND METHODS

Animals, nematode infection, and autopsy

Specific pathogen-free 8-wk-old female rnu/rnu (F344/N Jcl-rnu) rats and their littermate rnu/+ (F344/N Jcl-rnu/+) rats were purchased from Clea Japan, Inc. (Tokyo, Japan). The animals were inoculated subcutaneously with 1,000 *N. brasiliensis* L3 larvae as described previously (Hyoh et al., 1999). Uninfected animals and animals that had been infected 10 or 20 days previously (n = 4 in each group) were killed by inhalation of ether. The whole small intestine was removed and divided into the following segments (expressed as the distance from the pyloric ring): 0–18 cm, 18–22 cm, 22–26 cm, and 26 cm to the end of the ileum. The 0- to 18-cm and 26-cm to ileum end segments were used for the worm count, the 18- to 22-cm segment for paraffin-embedded tissue sections, and the 22- to 26-cm segment for separation of the epithelium, as described in the following sections.

Tissue preparation for histology

The segment of the jejunum 18–22 cm distal to the pyloric ring was opened longitudinally, fixed in 4% buffered formalin overnight, and embedded in paraffin in such a position that histological sections could be cut perpendicular to the luminar surface.

Morphometry

Measurements were carried out on hematoxylin and eosin–stained sections cut at 5 μm. Twenty villus–crypt units (VCU), which were cut as nearly perpendicularly as possible, were selected per animal, and the lengths of the villi and crypts were measured directly under a microscope using an ocular lens with a micrometer. The average length of 20 VCU was used as the representative length in a given animal, and the

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means and standard errors of 4 animals were calculated. The surface to volume ratio was measured as described by Dunnill and Whitehead (1972). In brief, sections were projected onto a white board, on which were drawn 15 lines of equal length, as described by Weibel (1963). The magnification was such that the length of each line corresponded to a length (L) of 1.4×10^{-2} cm. The section was projected at random onto the template lines, and the number of times the lines cut the mucosal surface (c) and the number of times the endpoints of the lines fell on mucosal tissue (h) were counted from 20 randomly selected fields per animal. The ratio c/Lh gives an index of the volume to surface ratio.

Worm counts

The numbers of worms in the intestinal segments of 0–18 cm and 26 cm to ileum end were determined by the saline incubation method. The numbers of worm profiles on the mucosal surface in the tissue sections 18–22 cm from the pyloric ring were also counted under a microscope.

Immunohistochemistry and cell count

Paraffin-embedded tissue sections were prepared as described above. The dewaxed sections were treated with 0.3% H_2O_2 for 20 min, immersed in 0.01 M sodium citrate buffer, pH 6.0, and then autoclaved at 121 C for 10 min for antigen retrieval according to the method described by Bankfalvi et al. (1994). The sections were incubated for 1 hr with anti–granzyme B or anti-CD3 goat IgG (Santa Cruz Biotechnology, Santa Cruz, California) or with anti-CD8 monoclonal antibody (OX-8) (Cymbus Biotechnology, Chandlers Ford, U.K.). For a negative control, species-matched normal IgG was used. After the sections were washed, they were incubated with horseradish peroxidase–conjugated anti-goat IgG (Nichirei Corp., Tokyo, Japan) or with anti-mouse IgG conjugated with peroxidase-labeled dextran polymer (Envision+, Dako, Carpinteria, California). The final reaction was carried out in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.2 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Dojin Lab., Kumamoto, Japan) and 0.005% hydrogen peroxide. The numbers of immunoreactive cells in 10 VCU were counted under a microscope, and the number per VCU was calculated.

Separation of intestinal epithelium

The separation of epithelium from the jejunum was carried out at 4 C in ethylenediaminetetraacetic acid (EDTA)–Hanks solution (Ca^{2+}-Mg^{2+}–free Hanks balanced salt solution supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.3, and 1.0 mM EDTA), as described elsewhere (Hyoh et al., 2002) with a slight modification. In brief, a piece of jejunum 22–26 cm from the pyloric ring was removed, opened longitudinally, and cut into segments 1 cm in length. After a brief wash in phosphate-buffered saline, 4 pieces of tissue were put into a 15-ml tube containing 4 ml of 1.0 mM EDTA-Hanks solution, and debris attached to the mucosal surface was removed by vigorously shaking the tubes 15 times by hand. The tissues were then transferred into another tube containing 1.0 mM EDTA-Hanks solution. After 75 min on ice with occasional agitation of the tissues by inversion of the tubes, the epithelial cells were separated by vigorously shaking the tube by hand 60 times. After the tissue was discarded, the detached epithelia were collected by centrifugation at 600 *g* for 3 min at 4 C, and the pellets were kept at −80 C until use. Giemsa staining of the separated epithelial fractions showed not only epithelial cells but also mononuclear cells, the majority of which we considered might have been intraepithelial lymphocytes (IEL). Histological examination of the tissue after collection of the epithelia showed that villus epithelia were separated completely, whereas epithelial lining cells in the lower part of crypts were still attached to the tissue in approximately half the crypts. In uninfected rats, the basal lamina of the epithelium was intact, and lamina propria cells were retained in the tissue, but in animals after 10 days of infection, the basal lamina was partly obscured, indicating that some propria mucosal cells contaminated the epithelial fractions.

Extraction of total RNA, complementary DNA synthesis, and polymerase chain reaction

Total RNA was extracted using TRIZOL Reagent (Life Technologies, Rockville, Maryland). Two-microgram aliquots of RNA were reverse transcribed in 20 μl of reverse transcription (RT) buffer containing 5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphate (dNTP) mixture, 1 U/μl ribonuclease inhibitor, 0.25 U/μl AMV reverse transcriptase, and 0.125 μM oligodT-adaptor primer (Takara RNA LA PCR kit, Takara Biomedicals, Osaka, Japan) at 42 C for 50 min. One-microliter aliquots of synthesized complementary DNA were added to polymerase chain reaction (PCR) buffer containing 2.5 mM $MgCl_2$, 0.2 μM dNTP mixture, 0.025 U/μl LA *Taq* DNA polymerase (Takara RNA LA PCR kit), and 0.2 μM sense and antisense primers in a final volume of 10 μl. PCR was carried out with cycles of 30 sec at 94 C, 30 sec at 62 C, and 30 sec at 72 C. The sense and antisense primers used were: 5′-AGAAGAGCTATGAGCTGCCTGACG-3′ and 5′-CTTCTGCATCCTGTCAGCGATGC-3′ for β-actin with a 236-bp product; 5′-GACTTTGTGCTGACTGCTGCTCAC-3′ and 5′-TTGTCCATAGGAGACGATGCCCGC-3′ for granzyme B with a 495-bp product; 5′-ATAGAGCTGTGGCTACCGGTG-3′ and 5′-CTCCAGAGATCAAAGCAGTTCC-3′ for FasL with a 286-bp product; and 5′-GAGTGACAAGCCCGTAGCCC-3′ and 5′-GCAATGACTCCAAAGTAGACC-3′ for TNF-α with a 441-bp product.

Density analyses of PCR products

Six microliters of the amplified product was electrophoresed on agar and stained with ethidium bromide. The fluorescence images were saved with an image saver (ATTO Incorporation, Tokyo, Japan), and the density of each band was analyzed with NIH Image software. To determine the optimal numbers of PCR cycles, the densities of the electrophoresed PCR product were analyzed after different numbers of PCR cycles. For semiquantitative analyses, band densities were normalized relative to those of β-actin as described elsewhere (Kuroda et al., 2002).

Statistical analysis

Student's *t*-test (2 tailed) was used for statistical analysis; a P value less than 0.05 was considered significant.

RESULTS

Comparison of intestinal epithelial injuries induced in euthymic and athymic rats after infection with the nematode *Nippostrongylus brasiliensis*

Villus atrophy, which is accompanied by compensatory crypt hyperplasia, is a hallmark of intestinal epithelial injury induced by intestinal parasite infections. To determine whether a thymus-dependent mechanism is involved in the development of intestinal epithelial injuries, athymic F-344 rnu/rnu rats and their littermate euthymic rnu/+ rats were infected with 1,000 *N. brasiliensis* L3 larvae. Gross inspection of the small intestinal lumen 10 days after infection showed that the worm population density was the highest in the upper part of the jejunum of euthymic as well as athymic rats, whereas few worms were found in the distal part of the small intestine. After the highly populated intestinal segment 18–26 cm from the pyloric ring was removed for tissue section preparation and separation of the epithelium, other parts of the small intestine were used to examine the worm burdens. The number of worms that emerged in the saline was significantly more in athymic than in euthymic rats 10 days after infection (Fig. 1a). Twenty days after infection, worms had been totally rejected from the euthymic rat intestine, whereas sustained infection was observed in athymic rats. Because worm counts were performed in intestinal segments where the worm population is low, we also counted the numbers of worm profiles observed in paraffin-embedded tissue sections that were obtained from the highly populated segment 18–22 cm from the pyloric ring. The number of worm profiles in tissue sections was also significantly more in athymic rats than in euthymic rats (Fig. 1b).

Histological examination of the proximal jejunum showed

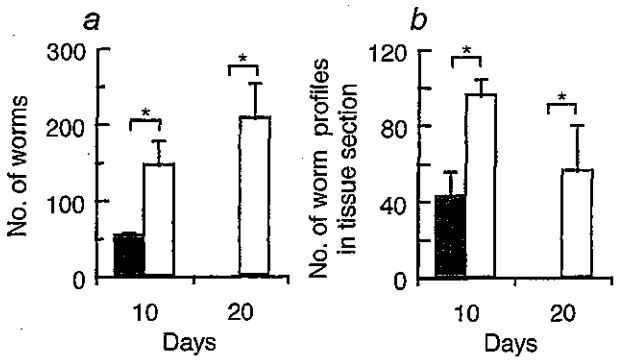

FIGURE 1. Worm burdens in the small intestine of rnu/+ (closed columns) and rnu/rnu rats (open columns) infected with 1,000 *Nippostrongylus brasiliensis* L3 larvae. (a) Number of worms in intestinal segments (expressed as the distance from the pyloric ring) 0–18 cm and 26 cm to the ileum end determined by the saline incubation method. (b) Number of worm profiles in 4-cm-long longitudinal sections of the proximal jejunum (18–22 cm from the pyloric ring) stained with hematoxylin–eosin. Columns and bars represent means + SE of 4 animals. * indicates $P < 0.05$.

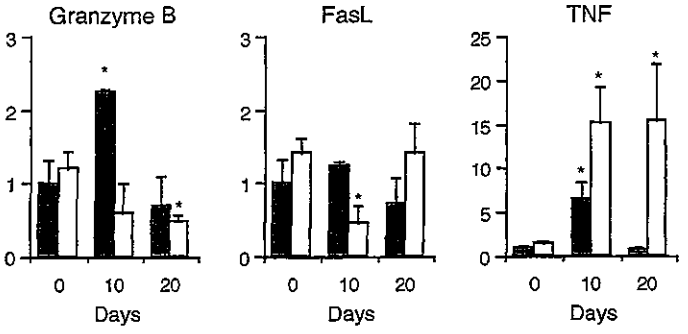

FIGURE 2. Expression of granzyme B, FasL, and TNF-α in the jejunum of rnu/+ (closed columns) and rnu/rnu rats (open columns) after *Nippostrongylus brasiliensis* infection. Total RNA was extracted from the jejunal epithelial fraction containing both epithelial and mononuclear cells, and RT-PCR was performed. The density of each PCR product was normalized with respect to that of β-actin. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with the day-0 average levels in rnu/+ rats expressed as 1.0. * indicates those significantly different from the day-0 level ($P < 0.05$).

marked cytopathic alterations in the villus epithelium 10 days after infection. In euthymic rats, villi were blunt in shape and epithelial cells were occasionally desquamated in a sheet from the basal lamina, leaving an eroded mucosa. Athymic animals rarely showed blunted villi, although at the villus tips small clusters of rounded epithelial cells with pyknotic nuclei were frequently found. In some villi of euthymic and athymic rats, the propria mucosa was markedly edematous with dilated lymphatic vessels.

Morphometric analyses revealed that the villus length in euthymic rats was significantly reduced 10 days after infection and returned to the preinfection level after 20 days, whereas villus atrophy did not develop in athymic animals despite the fact that the nematode infection persisted until 20 days after infection (Table I). The surface to volume ratio, an index of villus surface area determined by the method described by Dunnill and Whitehead (1972), also showed a significant reduction in euthymic rats 10 days after infection, but not in athymic rats. Crypt length reflects the cell growth activity in the crypt growth zone, and it increases significantly in response to the villus epithelial cell loss. Although there was no significant alteration of villus length or surface to volume ratio in athymic rats, crypt length was increased in athymic rats as markedly as in euthymic animals. These results suggest that epithelial injuries and cell loss occurred in both euthymic and athymic rats, but that the severity of the injury in athymic rats might not have been sufficiently intense to cause the villus atrophy that developed in euthymic rats.

Upregulation of cytotoxic factors in euthymic and athymic rats after infection with the nematode *Nippostrongylus brasiliensis*

Granzyme B, FasL, and TNF-α, which are expressed mainly in CTL, NK cells, or macrophages, are important mediators or ligands that can induce target cell injury (Green, 1998). We examined granzyme B, FasL, and TNF-α messenger RNA expression in the isolated jejunal epithelial fraction, which is composed of epithelial cells and IEL, by RT-PCR. The granzyme B expression level in euthymic rats was increased significantly 10 days after infection and decreased to the preinfection level 20 days after infection, whereas that in athymic animals showed no significant increase after infection (Fig. 2). The FasL expression level in euthymic animals did not change significantly after infection, whereas that in athymic animals was decreased after 10 days. The TNF-α expression level in euthymic rats was increased after 10 days and decreased to the preinfection level after 20 days, whereas that in athymic rats was increased after 10 days and maintained at a high level through 20 days after infection.

TABLE I. Morphometric analyses of jejunal mucosa of euthymic rnu/+ and athymic rnu/rnu rats infected with *Nippostrongylus brasiliensis*.*

	Villus length (μm)		Crypt length (μm)		Surface–volume ratio (c/Lh)	
Day	rnu/+	rnu/rnu	rnu/+	rnu/rnu	rnu/+	rnu/rnu
0†	447.9 ± 7.4	444.6 ± 9.1	163.8 ± 10.5	166.3 ± 10.7	50.8 ± 2.2	42.7 ± 2.3
10	332.9 ± 29.4‡§	474.0 ± 16.0	368.0 ± 7.0‡	360.7 ± 8.4‡	24.3 ± 2.1‡§	45.9 ± 2.7
20	433.0 ± 19.8	417.0 ± 10.5	235.5 ± 12.4‡	261.3 ± 16.5‡	32.1 ± 1.7‡	39.5 ± 5.4

* Each measurement was performed on paraffin-embedded tissue sections. Surface–volume ratio was determined as described in the Materials and Methods. All data are mean ± SE of 4 rats.
† Uninfected control.
‡ Significantly different from rnu/rnu rats ($P < 0.05$).
§ Significantly different from day 0 ($P < 0.05$).