表2　インフリキシマブ中止による薬剤投与の変化と中止時の検査値

症例	インフリキシマブ投与前	インフリキシマブ（中止時）	RAPA（中止時）	CRP mg／dl（中止時）	MMP-3 ng／ml（中止時）
K.S.	MTX 6 mg	MTX 4 mg	40未満（−）	0.03	47.3
T.A.	MTX 4 mg，NSAID	MTX 6 mg	40	0.05	51.1
S.M.	MTX 4 mg，ブシラミン，PSL 1 mg	MTX 2 mg	40	0.01	37.5
Y.H.	MTX 6 mg，PSL 5 mg，NSAID	MTX 4 mg	40未満（−）	0.01	39.8
M.M.	MTX 6 mg，PSL 5 mg	MTX 4 mg	40	0.21	56.3
M.N.	MTX 6 mg，PSL 5 mg	MTX 2 mg	40未満（−）	0.11	94.2
D.M.	MTX 6 mg，NSAID	MTX 4 mg	40	0.19	40.7
S.T.	MTX 6 mg，NSAID	MTX 6 mg	40未満（−）	0.08	34.7

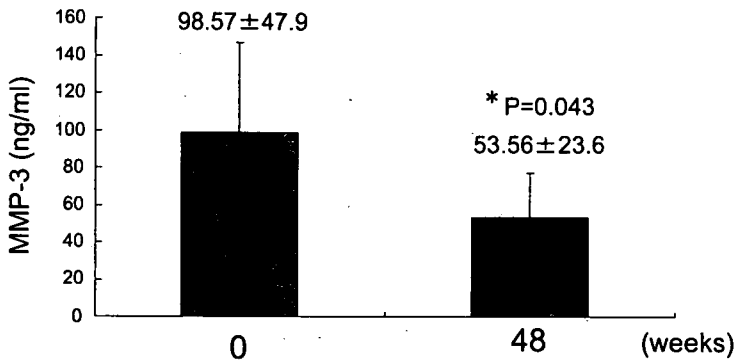

図5　寛解症例の投与後1年のMMP-3の変化

いほど，あるいは年齢が高いほどインフリキシマブは脱落する症例が多かったことを示す。寛解症例のMMP-3は投与前98.57±47.9ng／mlから53.56±23.6ng／mlに有意に減少を示した（P＝0.043）（**図5**）。寛解症例の経時的CRPの推移を見ると，症例5以外の全例はCRP陰性が継続しているが，症例5は投与中止して18週後よりCRP 0.45mg／dlと陽性化したがMTX 4 mgのまま継続し，その8週後に再び陰性化した（**図6**）。症例4は中止後1年でMTXを中止してCRPは陰性化を継続しており治癒の可能性がある。DAS28は投与前平均6.1（5.07〜7.55）が投与中止後平均2.06（1.88〜2.3）に低下していた。手ＸＰにおいて8例とも骨萎縮が改善し，症例5においては骨びらんの改善が投与中止後1年にて継続して認められた（**図7**）。インフリキシマブ投与前の薬剤は寛解例8例のうちステロイドを服用していた4例は，全例ステロイドを中止できMTXと葉酸にて治療継続している（**表2**）。

考　　察

RAの寛解については1948年Shortらによってはじめて報告されて以来，寛解基準については多くの議論がされているが，現在ではDAS28が2.6以下を寛解基準と決めている場合が多い[8]。しかし，抗リウマチ薬のみでの寛解は臨床上寛解でもMRI上，滑膜炎が持続して

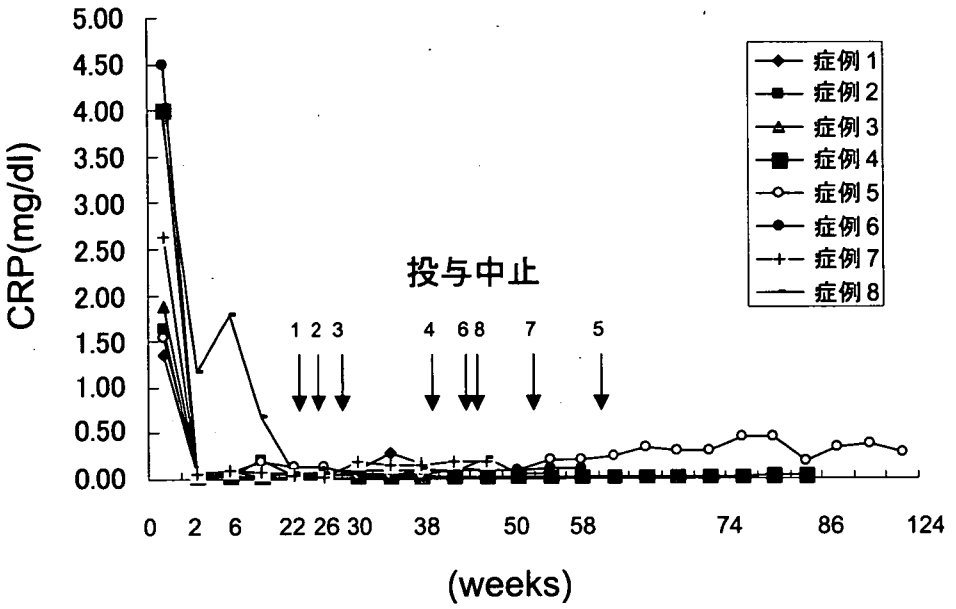

図6　寛解症例のインフリキシマブ投与中止後のCRPの推移

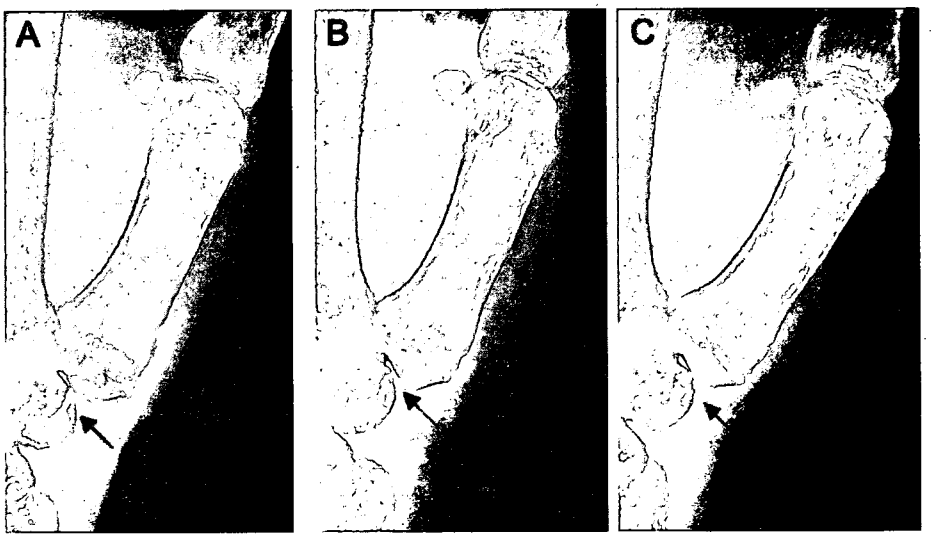

図7　インフリキシマブ中止できた寛解症例のXPの変化
A：投与前，B：投与後1年，C：中止後1年，矢印：骨破壊修復

いることが報告されており，DAS28のみでの寛解基準には疑問視される点もある[2,5]。現在のところ生物学的製剤を中止できる寛解についての基準はなく，本論文は本邦において初めての報告である。われわれは中止できる寛解基準をDAS28が2.6以下およびCRPが完全に陰性化して6ヵ月以上経過したものとした。この基準は今後本症例の長期経過観察において改良する可

表3：インフリキシマブの継続因子分析（Cox回帰分析の結果）

	B	標準誤差	Wald	自由度	有意確立	Exp (B)	Exp (B) の95.0% CI	
							下限	上限
CRP 前	−0.831	0.407	4.168	1	0.041	0.436	0.196	0.967
CRP 後	1.011	0.530	3.635	1	0.057	2.747	0.972	7.764
年　齢	0.243	0.115	4.472	1	0.034	1.274	1.018	1.596
罹患期間	−0.053	0.054	0.950	1	0.330	0.949	0.853	1.055
MTX	−0.426	0.302	1.990	1	0.158	0.653	0.362	1.180
steroid	0.184	0.166	1.234	1	0.267	1.202	0.869	1.664

能性があるが，少なくとも中止するまでの期間が長ければ長いほど患者に対する副作用や経済的負担が大きくなる可能性がある。Quinnらは早期RAに対してインフリキシマブを中止して1年後のMRIで滑膜炎が抑制され，2年後も機能的に効果が持続していたと報告している[7]。われわれの症例ではCRPだけでなくRAPAとMMP-3すべてが陰性化していないと再燃する可能性があることを経験した。インフリキシマブにおいて寛解による中止症例は本症例のごとく存在するが，エタネルセプトにおいて報告がないのはエタネルセプトにおいてCRPの陰性化が持続してもRAPAかMMP-3どちらか一方が陰性化していない症例が多かったためと筆者らは考えている。よって生物学的製剤の中止後再燃しない指標としてCRP，RAPAおよびMMP-3の3つが全て陰性が重要と思われる。

RAの治療はこれまでピラミッド式に行われることが多く、特に早期RAに対してはNSAIDやBucillamineなどで2～3年経過を見ることが多い。手指の変形が始まって初めてMTXなどの免疫抑制剤が使用されるが，間質性肺炎などの副作用の問題で使用されない症例もある。薬物治療ではClassの進んだRAにおいてはすでに肺などの全身の合併症を伴うことが多く，無理にMTXを増量したりすると心不全や肺炎を引き起こす危険性は高い。生物学的製剤はいかなる薬剤にも抵抗性のある症例に使用すべきと考えられる傾向があるが，その理由の一つとして生物学的製剤による副作用が重篤であるかもしれないとの医師側の危惧による。しかしながら，生物学的製剤のRAに対する適応を再検討すると，MTXに効果不十分な症例に限らず，早期RAにおいて少量のMTX（当科では4 mg／週）とインフリキシマブを用いて早く治療を行い，早く寛解に移行できれば生物学的製剤が中止可能であり，副作用の危険性はより少なくなる。薬物治療は長期間投与により蓄積された薬物の副作用と身体に与える負の影響を考慮すれば，最善の治療は薬剤中止できる治療と言える。今回，当科においてインフリキシマブで治療した193例のうち3年での継続率は48%であった。すなわち3年で約半数は脱落することを考慮すると，生物学的製剤は長期に使用するためのものでなくむしろ3年以内に中止できるような症例に使用すべきであると考える。これらは発症早期RAであり，当科では25%の高い寛解導入率を示した。

すなわち生物学的製剤の最もよい適応は早期RAに対する寛解導入療法であり，RAを早く発見して早く治療する最適の治療手段として力を発揮できる。早期RAに対しては海外でもインフリキシマブを用いて骨破壊を抑制したり，寛解をもたらすとの報告がある[9]。さらにはインフリキシマブを中止できた寛解の報告があるが，その詳細は明確ではない[3]。今回，われわれはインフリキシマブを中止できた寛解を追跡調査しStage Ⅲの症例5では投与中止して18週後より一時的にCRPが陽性化しており，この点に中止できうる寛解治療の限界がある可能

性がある。すなわちStage Ⅱ以下の手指の変形のないRAにおいてはこの寛解導入療法で安定したCRP陰性化をもたらすことができることが示唆される。今後5年以上の追跡調査が必要と思われる。

早期RAの診断では従来の診断基準においてもすでに進行していることがあり，当科では発症5年以内で手指MPあるいはPIPの腫脹と圧痛があり，CRP 0.5mg／dl以上，XPにて骨びらんあるいは骨萎縮を認め，両手に朝のこわばりがあるものを早期RAと診断し，Key drugとなるMTX 4 mgを開始する。患者と十分，RAの骨破壊について相談し，生物学的製剤を早期に開始する。自然寛解の可能性についても考慮するが，長期間経過観察しなければいけない点と，現在のところ本当に一生患者が自然寛解を継続できるかのデータはなく，経験上一時的に自然寛解に入っても3～5年すれば再びCRPやリウマチ因子が陽性になり，骨破壊が進む症例があることから自然寛解のエビデンスが確実でない以上，こうした中で少なくとも生物学的製剤により骨破壊抑制がもたらされ，CRP陰性，リウマチ因子陰性，MMP-3も陰性化できる寛解導入治療が現在存在することは患者側にとって十分治療のメリットがあるといえる。

生物学的製剤の効果減弱例に対して薬物を交替するよりもRAの病態の場である滑膜を除去して，サイトカイン産生を抑制し，生物学的製剤の効果を再現できることを筆者らは報告した[4]。寛解導入療法において今後Stage Ⅱ以下の関節破壊の少ないRAにおいて生物学的製剤の効果減弱例であっても関節鏡視下滑膜切除により寛解導入率が増加できる可能性があり整形外科的治療と生物学的製剤による集学的治療が発展する可能性がある。

ま と め

関節リウマチに対する生物学的製剤インフリキシマブによる寛解導入療法を行ない8例のインフリキシマブを中止した寛解の臨床経過を追跡調査した。Stage Ⅲの症例は中止後CRPの陽性化を一時的に認めたが，Stage Ⅱ以下の症例でインフリキシマブ中止時にCRP,リウマチ因子，MMP-3すべてが陰性化している症例は再燃を認めなかった。また寛解導入療法にてインフリキシマブ中止後1年でMTXも中止できた症例もあり，これは関節リウマチの治癒達成にむけた一歩前進した治療とも考えられ今後の長期報告が期待される。

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骨関節破壊の進行とQOL

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関節リウマチ(RA)の病態の中心は関節機能障害であり，骨関節破壊により進行する。骨関節破壊の進行は各症例により一律でないが，発症5年以内に75%が起こるとされている。RAの早期からの骨関節破壊を，治療によりいかに最小限に抑えるかが患者のquality of life (QOL) につながる。近年，生物学的製剤の治療によりRAの骨関節破壊修復について注目されている。我々の臨床研究では，早期RAで関節破壊が乳頭状滑膜と有意に関係していることが認められている。この乳頭状滑膜増生をいかに抑えられるかがRAの骨関節破壊を食い止める鍵となる。骨関節破壊の抑制は，生物学的製剤で効果減弱した症例などの増生した乳頭状滑膜を除去することにより確実なものとできる。今後，生物学的製剤の骨関節破壊抑制について詳細な解析が必要である。これにより患者QOL向上により具体的な治療選択が明解となる。

Progression of bone and articular destruction and QOL.

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The joint dysfunction caused by bone and articular destruction is the most important pathology in rheumatoid arthritis (RA) patients. The progression of bone and articular destruction starts within five years from disease onset, however depending each case inflammation. Quality of life (QOL) of RA patients needs early stage treatment to prevent joint destruction. We recognized villonodular synovial proliferation is significantly correlated with early stage RA. Therefore to decrease those synovium leads to prevent joint destruction. Biological therapy itself can not inhibit villonodular synovial proliferation in effect attenuation cases. Arthroscopical synovectomy is effective to remove those synovium and restore the effect of biological therapy. It is needed that detail analysis of improvement of joint destruction by biological therapy near future to lead the improvement QOL of RA patients.

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はじめに

近年，関節リウマチ（RA）の治療が生物学的製剤により大きく変化してきている中で，その骨関節破壊の抑制効果は関節機能障害を予防しquality of life（QOL）改善につながる。しかし生物学的製剤を使用してもすべての症例で有効なわけではなく，未だ骨関節破壊抑制の詳細な解析はなされていない。日本においてどのような症例に骨関節破壊抑制効果があるか，詳細な検討が必要であると思われる。RA自体が単一なものでなくさまざまなstageにおいて骨関節破壊の程度が違っており，QOLを考える意味で生物学的製剤使用のみでは現在のところ不十分である。リウマチ治療薬と合わせて，関節鏡視下滑膜切除や人工関節等の手術療法にて関節機能障害を改善することにより，患者のQOL向上を目指す。以下，生物学的製剤による骨関節破壊のQOL改善だけでなく，手術療法についても述べる。

骨関節破壊の進行

Masiらは初期RAを5年間経過観察した報告によると，RA患者の経過は①炎症が数年間で鎮静化し，骨破壊も進行しない単相型（monocyclic）20%，②炎症の増悪を繰り返しながら経過し，骨破壊も進行する多相型（polycyclic）70%，③炎症が高く，骨破壊が進行性に経過し，寝たきりになっていく進行型（progressive）10%の3つの型に分けられると報告している[1]。メトトレキサート（MTX）による内服治療においても，関節破壊は現在のところ止めることはできない（図1）。

Yamamotoらは，発症1年以内の患者を2年間観察して，2年以内に寛解となる群15%，治療によって日常生活には不自由のない群50%であり，この2つの群の合計である65%は治療によ

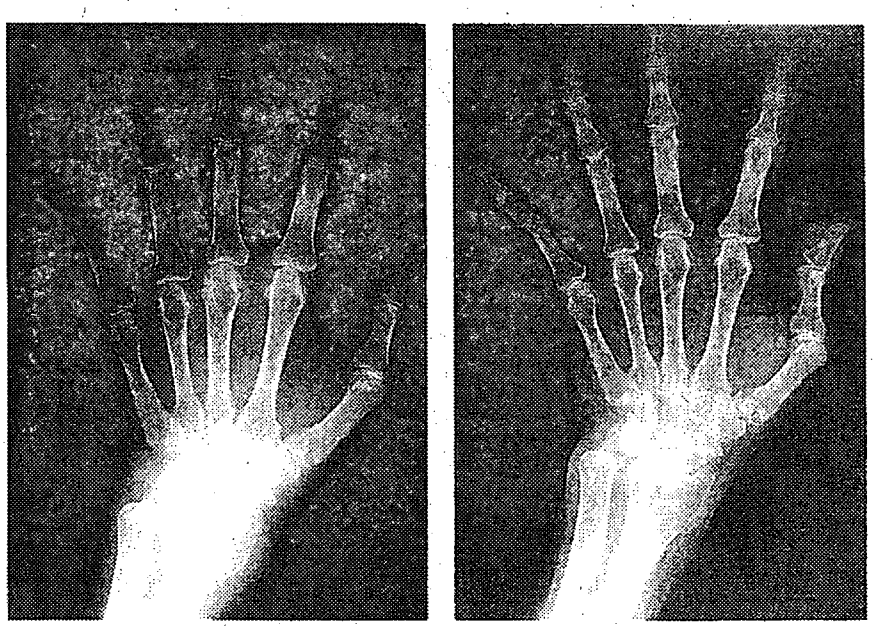

図1　関節破壊の進行

MTX内服のみでは関節破壊の進行は止めることができない。

（筆者ら提供）

MTX：メトトレキサート，QOL：quality of life，RA：関節リウマチ

り十分コントロールできるのでcontrol群と分類している[2]。治療するにもかかわらず身体機能障害者となるprogressive群は35%でuncontrol群とした。

Ochiらは，罹患期間10～15年の240例の破壊関節数の分布より，RA患者の関節破壊の広がりの自然経過に基づく病型を3つのタイプに分類した[3]。Ⅰ群小関節破壊型病型（LES）65%，Ⅱ群多関節破壊型病型（MES）30%，Ⅲ群ムチランス型病型（MUD）5%とし，Ⅱ，Ⅲ群には明らかな寛解はないと報告している。日常生活において，この患者がどの病型に属し，将来とるであろう経過を予測し，どのような薬物治療を選択するか，手術の時期の決定を早くするかどうかが重要な問題点である。

RAの骨関節破壊の原因として考えられるのは，病態の場が関節を中心としており滑膜増生および全身の免疫学的異常にかかわることである。そこで我々は関節滑膜の性状の病的変化に注目すべきと考え，17例の関節滑膜切除時に採取した滑膜所見を，組織学的に滑膜増生，乳頭状，血管増生，フィブリン析出，リンパ球浸潤の5項目に分類し，患者の罹患期間を従属変数にとった重回帰分析により，乳頭状滑膜が早期RAと有意に関係していることをつきとめた（$p = 0.018$）。関節破壊を起こす時期は発症して5年以内に75%であるとされることから，罹患期間の少ない時期に多く見られるこの乳頭状滑膜に骨関節破壊の原因があることも理論的に考えられる。

しかしながら，乳頭状滑膜がどのようにして骨関節破壊を起こすかは，サイトカインやケモカインおよびmatrix metalloproteinase（MMP）などの液性因子[4]のほか，滑膜内の破骨細胞の存在あるいは分化促進，さらには形態学的に関節周囲の関節包付着部の軟骨欠損部いわゆるベアエリアに入り込みやすいことも考えられる。浸入した乳頭状滑膜がやがてベアエリアを骨びらんへと進展させ骨関節破壊を促進する。

RAが発症して早期の関節で，C-reactive protein（CRP）上昇が持続する症例では特に，乳頭状滑膜の増生がみられる。さらに，RAが進行していても関節変形が少なく骨破壊が少ない関節で，CRPが上昇している症例では乳頭状滑膜が見られる。すなわち乳頭状滑膜は，発症罹患期間に統計学的には有意に関係するが，特に個々の関節破壊進行の早期と関係があると考えられる。よって骨破壊をくいとめて個々の関節破壊進行をとめるには，乳頭状滑膜増生を抑制することが鍵となる。

生物学的製剤を用いた骨関節破壊抑制効果は手足の小関節に比較的よく見られるが，時に膝などの加重関節にも認められることもある（**図2**）。

QOLからみた経過と予後

橋本らはArthritis Impact Measurment Scales, version 2（AIMS2）日本語版調査表を作成し，それを用いて11施設RA患者1,774例を調査した[5]。患者QOLに影響を及ぼす主要因子として，筋力，関節，慢性病期，貧血，炎症の因子の順で関与していた。また，MTX療法と過去2年以内の人工関節置換術は，QOLの改善に有意に貢献した。

居村らは，下肢大関節に人工関節置換を受けた患者358名のQOL調査を行った[6]。患者のQOLは人工関節置換術を受け改善されるが，長期的に見れば，手術以外の治療に負うところが大きい。また，ムチランス型破壊，2椎体以上の圧迫骨折の存在がQOLに大きな影響を持つと報告した。

患者の日常生活の中で，四肢筋力を保持させる運動や生活方法，在宅でのリハビリテーションの

AIMS2：Arthritis Impact Measurment Scales, version 2，CRP：C-reactive protein，LES：小関節破壊型病型，MES：多関節破壊型病型，MMP：matrix metalloproteinase，MUD：ムチランス型病型

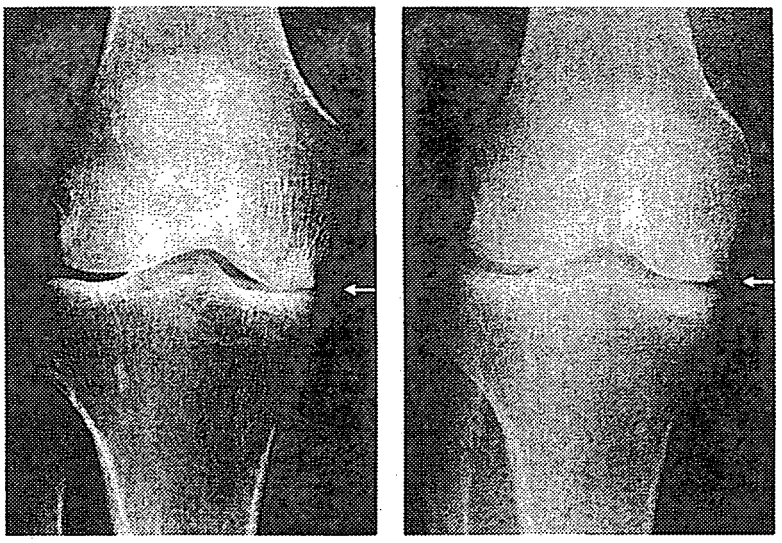

図2　骨関節破壊の改善

膝関節内側における関節裂隙の改善と，脛骨の骨萎縮の改善を認める。
（筆者ら提供）

指導，リハビリテーション施設の利用などの生活指導が必要である。また，薬物治療による炎症コントロールや下肢大関節の関節置換術の適応の有無を判定し，手術時期を逸しないように患者に伝えることが必要である。

生物学的製剤使用中の関節鏡視下滑膜切除の効果

生物学的製剤投与中に，患者本人から関節痛の痛みが緩和されたとの声をしばしば聞くことがある。しかしながら，膝や肩などの大関節においてはどうしても疼痛がとれないこともあり，こうした患者に対しては関節鏡視下滑膜切除術を行っている。これは，滑膜からのサイトカイン産生を減少させ骨関節破壊を抑制する意味がある。

インフリキシマブ投与にても改善が認められない関節滑膜では，血管増生を伴った乳頭状の滑膜が見られる（**図3**）。インフリキシマブ投与中における関節鏡視下滑膜切除術後の成績は良好で，CRPだけでなくdisease activity score 28（**DAS28**）の改善を導く。さらに，関節鏡視下滑膜切除術によって次回投与のインフリキシマブの効果が増強され，効果減弱例に対して有効な手段となり得る[7]。こうした生物学的製剤と手術療法の併用は，特に整形外科医にとって新しいRAに対する治療法として今後発展していく可能性がある。

生物学的製剤使用中の人工関節置換術

骨関節破壊の進行したRAに対して，生物学的製剤を使用して炎症症状は改善しても，既に進んだ関節破壊は改善することはできない。従って，人工関節手術の前に全身の炎症症状を抑えるという点では，生物学的製剤は有効な治療法である。ただし，間質性肺炎やインフュージョンリアクションなどの副作用を十分考慮しなければならない。初診時にCRP高値の患者と高齢者は，インフリキシマブの継続率が悪いことが分かっている。

DAS28：disease activity score 28

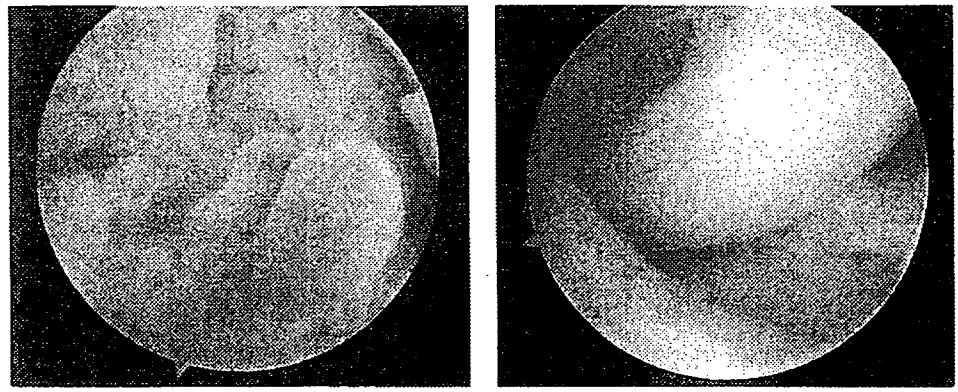

図3　インフリキシマブ効果減弱例の関節鏡視下滑膜切除術

膝関節内の血管新生に富む乳頭状滑膜を認めた。滑膜切除により再びインフリキシマブの効果が持続できる。（筆者ら提供）

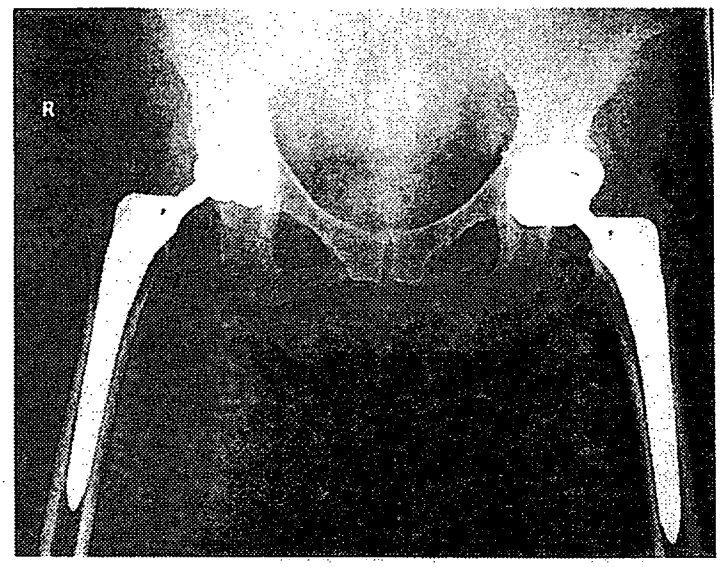

図4　インフリキシマブ投与中の人工股関節置換術

感染の危険性上昇なく，通常の手術と同様に行った。（筆者ら提供）

人工関節には足趾，足，膝，股関節，肘，肩，手指関節があるが，関節部位と生物学的製剤使用の特別な注意点と言えばやはり術後感染であり，これには十分注意を要することは言うまでもない（**図4**）。人工関節には生物学的製剤を使用したからといって通常の人工関節手術と特別な違いはないが，糖尿病との合併患者には注意を要する。

ただし，現実には生物学的製剤と人工関節置換術の術後感染の割合は非常に低く（東京女子医科大学東医療センター整形外科において43例中2例4.67%；1例足趾形成術後表層感染，1例脊椎後方固定術の血腫形成で軽微なもの），その理由として以下のことが考えられる。

RAに対する人工関節置換術の術後感染の比率は，血清CRPに比較的依存する。また，長期ステロイド使用患者には免疫力低下のため術後感染

に注意を要する。ところが生物学的製剤を使用すればステロイドなどを使用しなくてもCRPは改善していてむしろ比較的感染しにくい状態であり，人工関節置換術をしやすい環境であるとも言える。さらに，手術の時期であるが総じて生物学的製剤のインフリキシマブの投与と投与の中間で，具体的には投与後4週で行う場合が多く，生物学的製剤の影響は受けにくいことも考えられる。これらの人工関節などの手術を併用することによって，関節破壊による機能障害を改善し，患者のQOL向上を導くことができる。

おわりに

RAの骨関節破壊の進行とQOLについて生物学的製剤による治療を踏まえて述べた。骨関節破壊の進行を止めるためには十分な生物学的製剤を熟知した使用方法が必要であり，特に早期RAにおいては関節変形を食い止める手段となり得る。骨関節破壊の進行が既に進んでしまって日常生活が困難な場合には，生物学的製剤は炎症のコントロールとして用いられるが，単独使用では限界がある。従って，関節鏡視下滑膜切除や人工関節にて関節機能障害を改善することにより，患者のQOL向上を目指す。このように，手術も考慮した集学的治療が現在のRA治療では必要である。

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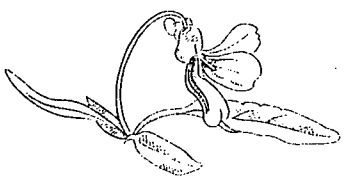

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BRIEF REPORT

Histological changes in bone marrow after treatment of infliximab for rheumatoid arthritis

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Abstract To investigate histological evidence of bone remodeling in response to infliximab for rheumatoid arthritis (RA), bone marrow tissues were extracted from ten RA patients at the time of total knee arthroplasty after treatment of infliximab for an average of 16 months (range, 8–24 months). The patients had a mean age of 65.3 years (range, 57–76 years) with 4.8 mg/week of methotrexate (MTX; 4–6 mg) and 3.8 mg/day of prednisolone (2–5 mg). Control samples were obtained from ten RA patients who did not undergo infliximab therapy. These patients had an average age of 67.6 years (range, 59–78 years) and received 5.2 mg/week of MTX (4–6 mg) and 4.0 mg/day of prednisolone (2–5 mg). Histological examination of structural differences between the infliximab and control groups in bone marrow was performed using hematoxylin and eosin (H & E) to evaluate differences. In immunohistochemical examination, the expressions of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), receptor activator of nuclear (kappa) B ligand (RANKL), osteoprotegerin (OPG), and osteopontin (OPN) were compared between both groups. H & E staining revealed that the bone marrow tissues of the RA patients who underwent infliximab therapy demonstrated newly formed thickness of interstitial septum among the trabeculae as compared with the control group. Moreover, immunohistochemical examinations revealed that TNF-α, IL-6, RANKL, OPG, and OPN were expressed in this newly formed bone after infliximab therapy. Therefore, treatment with infliximab improved the histological changes with respect to bone metabolism in the newly formed bone marrow tissues.

Keywords Bone · Histology · Infliximab · Rheumatoid arthritis

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Introduction

Recently, anti-tumor necrosis factor-α (TNF-α) therapy has been used in patients with rheumatoid arthritis (RA) to control inflammation and inhibit bone and joint destruction associated with methotrexate (MTX) therapy. Infliximab is an anti-TNF-α monoclonal antibody that blocks TNF-α for inhibiting cytokines and synovial inflammation [1]. Several studies have reported changes in bone metabolism after the use of infliximab [2, 3]; however, there has been no description of histological changes after infliximab therapy despite improvement seen on X-ray or magnetic resonance imaging [4]. Some patients with RA have shown improvement in bone atrophy or erosion in early RA in X-rays and improvement in bone absorption of elements such as N-telopeptide of type I collagen (NTX) or bone alkaline phosphatase (BAP) [2]. However, X-ray examination does not reveal why bone atrophy or erosion is improved. To assess the mechanism of improvement in bone metabolism, we performed histological examination in bone marrow obtained from patients who underwent total knee arthroplasty (TKA) after receiving infliximab therapy. On the other hand, several anti-TNF therapies have been evaluated recently; these include etanercept (a fusion protein consisting of the extracellular ligand-binding domain of the 75-kDa receptor for TNF-α and the constant portion of human IgG_1) [5], adalimumab (a fully human anti-TNF-α monoclonal

antibody) [6], tocilizumab (anti-interleukin-6 [IL-6] receptor) [7], rituximab (anti-cluster of differentiation 20) [8], and abatacept (cytotoxic T-lymphocyte antigen 4-immunoglobulin) [9] for treatment of RA. Based on the hypothesis that TNF-α therapy induces bone remodeling, we conducted an immunohistochemical study of bone marrow to analyze the expression of bone metabolic markers including RANKL, OPG, and OPN along with TNF-α and IL-6. This study presents the histological changes observed in bone marrow after the use of infliximab. This is the first report of histological evidence that infliximab induced bone healing.

Materials and methods

We treated 203 RA patients with infliximab from August 2003 to March 2007 in our hospital in Japan. After infliximab therapy for an average of 16 months (range, 8–24 months), bone marrow tissues were extracted with informed consent from ten RA patients at the time of TKA, which was performed because of severe joint destruction with pain. The disease duration was an average of 11 years (7–21 years) and patients had a mean age of 65.3 years (range, 57–76 years), one male and nine females. They received 4.8 mg/week of MTX (4–6 mg) and 3.8 mg/day of prednisolone (2–5 mg). According to Steinbrocker criteria [10], the infliximab group was classified as follows: class III, seven patients; class IV, three patients. Control samples were obtained from ten RA patients who did not undergo infliximab therapy. These control patients had an average age of 67.6 years (range, 59–78 years), two males and eight females, and received 5.2 mg/week of MTX (4–6 mg) and 4.0 mg/day of prednisolone (2–5 mg) at the time of TKA, with informed consent. The disease duration of control groups was an average of 12.2 years (6–19 years). According to Steinbrocker criteria, the control group was classified as follows: class III, seven patients; class IV, three patients. All patients who took part in this study were diagnosed according to the criteria of the American College of Rheumatology [11]. All samples of both groups were taken from medial condoyle at 2 cm from knee joint surface for same condition of taking place in bone marrow. None of the patients had a history of hormone replacement therapy (estrogen) nor had undergone bone-sparing therapy, including bisphosphonate or calcium supplements. Infliximab was administered by intravenous infusion at a dose of 3 mg/kg at baseline, then at 2 and 6 weeks, and then every 8 weeks. The clinical findings were as follows: urinary NTX, 58.5±4 nM bore collagen equivalent/mM Cr (nM BCE/mM Cr) in the infliximab group and 73.4±8 nM BCE/mM Cr in the control group; and BAP, 14.4±4 U/l in both the infliximab group and 13.1±2 U/l in the control group. X-ray examination of the knee joint was performed before and after infliximab treatment and results from the two groups were compared at the nonoperative site. Serial paraffin sections of the bone marrow (5 μm) were stained with hematoxylin and eosin (H & E). For immunohistochemical examination, the tissue sections were blocked for 10 min in phosphate-buffered saline containing 20% rabbit serum and then incubated overnight at 4°C with the following antibodies: anti-human osteopontin (OPN) mouse monoclonal antibody (1:250 dilution; Novocastra, Newcastle, UK), anti-human osteoprogerin (OPG; H-249) rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology, CA, USA), anti-human receptor activator of nuclear (kappa) B ligand (RANKL; FL-317) rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology, CA, USA), anti-TNF-α mouse monoclonal antibody (1:1,000; Biogenesis, Pool, UK), and anti-human IL-6 rabbit polyclonal antibody (Rockland Inc., Gilbertsville, PA, USA). After treatment with the second antibody, we compared the expression pattern of TNF-α, IL-6, RANKL, OPG, and OPN with that of the control group. Disease Activity Score 28 (C-reactive protein) levels [12] were also compared between the two groups at the time of histological examination. For microscopic evaluation, in each patient studied, a minimum of two different bone marrow tissue samples were examined. Microscopic evaluations were performed as described before [13] by one observer (KK) who was blinded to the name of patients. Cytokine and bone metabolic marker staining were assessed by estimating the number of positively staining cells in the ten different areas in bone marrow. All sections (minimum of two different bone marrow samples per patient) were examined and results were expressed as a mean for immunohistochemistry. Positivity was noted when complete staining of the cells was observed. Thickness of interstitial septum (TIS) in bone marrow was estimated by assessing all visible thickness of interstitial septum with cells in all bone marrow samples at a magnification of ×200 by H & E. Ten random readings per high-power field were recorded and the results expressed as a mean percentage (positive fields out of ten random fields) with standard deviation (SD) in each sample. Statistical analysis was performed using the Mann–Whitney U test for continuous variables and the chi-square test for categorical variables between the two groups. P values of less than 0.05 were considered significant.

Results

H & E staining revealed that the bone marrow tissues of the RA patients who underwent infliximab therapy demonstrated newly formed TIS, along with cell proliferation among the trabeculae (Fig. 1a,b). The TIS was significantly increased in the infliximab group as compared with the control group [1.7(0.5) vs. 85(7.2) respectively; p=0.024]

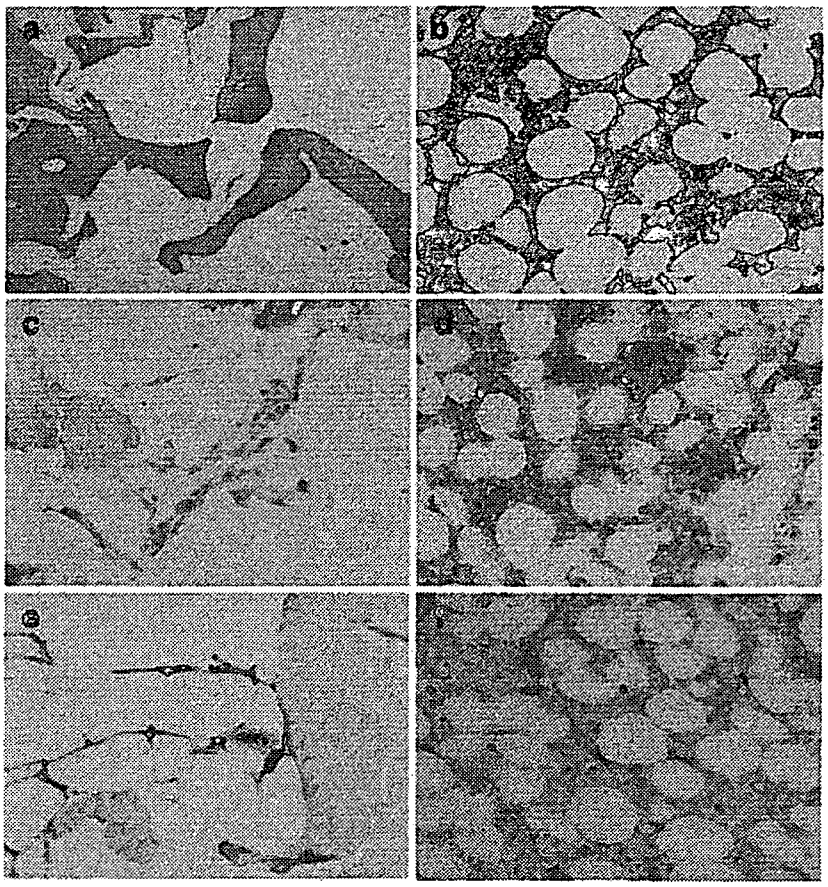

Fig. 1 Control group: **a**, **c**, **e**; infliximab group: **b**, **d**, **f**; H & E: **a**, **b**; TNF-α: **c**, **d**; IL-6: **e**, **f** (magnification ×200)

(Table 1); however, control group demonstrated thin septum of bone marrow with few cells. The immunohistochemical examinations revealed that TNF-α was increased in proliferation cells of TIS in infliximab group; on the other hand, control group was also positive in few cells. IL-6 was positive in fibroblastic cells of TIS in infliximab group and control group was also positive in few cells (Fig. 1c–f). In control group of IL-6, positive density was stronger than TNF-α. RANKL was increased in TIS in infliximab group; however, control group showed negative results (Fig. 2a,b). OPG was positive in several cells in TIS in infliximab group compared with no staining in control group (Fig. 2c,d). OPN was positive in TIS in infliximab group more strongly than in the control group (Fig. 2e,f). The numbers of positively staining cells of TNF-α, IL-6, RANKL, OPG, and OPN in TIS were increased significantly compared with the control [mean (SD) 41(3.7), 37(4.5), 32(4.6), 41 (3.3), and 36(4.3) vs. 17(4.5), 14(2.6), 3.6(0.7), 2.5(0.5), and 3.5(0.1) respectively; $p<0.05$] (Table 1). X-ray examination revealed that bone atrophy improved at the nonoperative site in eight of the ten patients and joint narrowing improved in only one of ten patients. The improvement of bone atrophy by X-ray almost paralleled with TIS-positive percent in infliximab group. In the infliximab group, NTX was significantly decreased compared with the control group ($p<0.05$). These findings provided the evidence that treatment with infliximab induced the histological changes of TIS formation in bone marrow with respect to bone metabolism for the newly formed bone tissue.

Table 1 Comparison of histological finding after treatment by infliximab

	TIS	TNF-α	IL-6	RANKL	OPG	OPN
Control ($n=10$)	1.7 (0.5)	17 (4.5)	14 (2.6)	3.6 (0.7)	2.5 (0.5)	3.5 (0.1)
Infliximab ($n=10$)	85 (7.2)*	41 (3.7)*	37 (4.5)*	32 (4.6)*	41 (3.7)*	36 (4.3)*

Results expressed as mean (SD) percentage of positive fields of TIS by H & E and positively staining cell numbers by immunohistochemistry
TIS Thickness of interstitial septum in bone marrow
*$p<0.05$

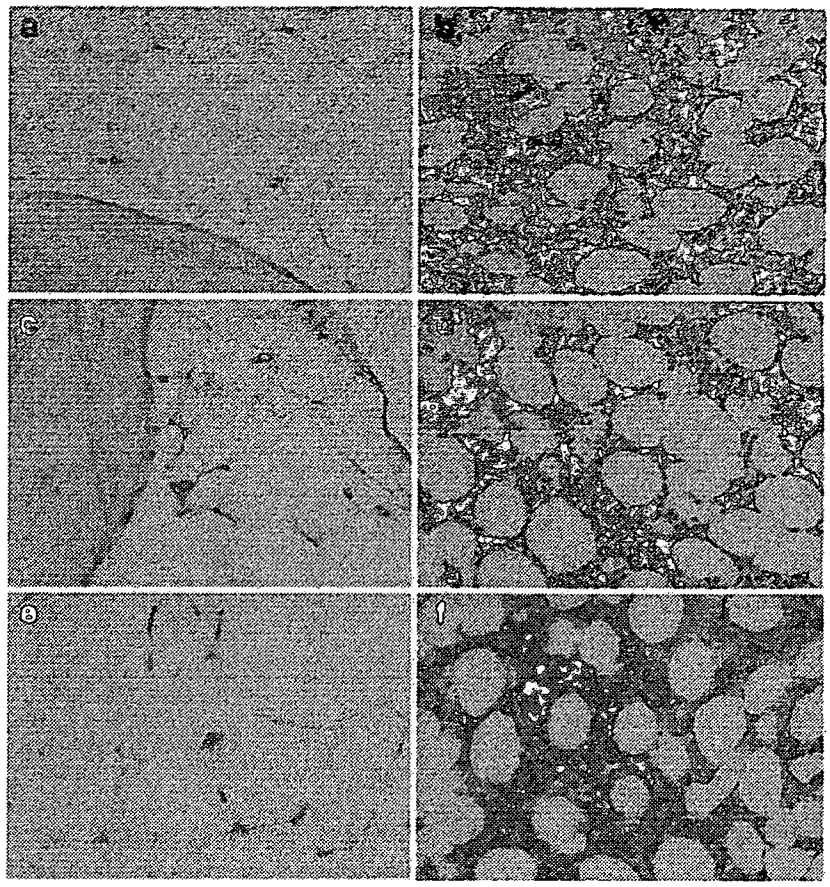

Fig. 2 Control group: a, c, e; infliximab group: b, d, f; RANKL: a, b; OPG: c, d; OPN: e, f (magnification ×200)

Discussion

It has been reported that bone remodeling of joint destruction occurred after undergoing the biological therapy, infliximab, for the treatment of RA [14]. However, histological examination was not performed to confirm the bone remodeling in terms of microstructural improvement. In this study, we detected TIS in fibrous bone tissue along with cell proliferation inside bone marrow using H & E staining, which showed TNF-α, IL-6, OPG, OPN, and RANKL. Because there is no fibrous bone tissue with cell proliferation in the bone marrow of control patients with RA, these cytokines and bone metabolic markers were not strongly found during immunohistochemical examination. RANKL, OPG, and OPN were detected in chondrocytes in deep layers of calcified cartilage near the subchondral region in normal bone [15]. OPG levels were increased in osteoblasts, RANKL levels were increased during differentiation of osteoclasts, and OPN levels were increased in hypertrophic chondrocytes in the deep layer of cartilage [15]. To achieve bone remodeling, it may be important to increase the metabolism of osteoclasts and osteoblasts for the formation of new bone marrow tissue. There was increased cell proliferation in bone marrow after anti-TNF therapy. Therefore, after infliximab therapy, bone remodeling was increased in terms of TIS and cell proliferation. It is difficult to understand the mechanism of change in cytokine production patterns in new tissue cells; however, histological confirmation of this mechanism is valid if new cytokine blockers are to be developed as biological agents for treatment of RA. In this study, X-ray examination showed that only bone atrophy was improved in large joints such as the knee after infliximab treatment in 80% of cases, which is the clearest evidence of bone improvement. Histological evidence of TIS in the newly formed bone marrow offers a possible explanation for the observed improvement in atrophy.

It has been reported elsewhere that bone metabolism markers, such as NTX and BAP, improved after 6 months of treatment with infliximab [2, 3]. NTX is considered to be a more sensitive marker than BAP or deoxypyridinoline, which are bone formation markers; however, its mechanism is still unknown. In our data, NTX was improved after infliximab treatment but BAP was not changed significantly. The histological change of TIS was found in all patients who received infliximab. Therefore, these bone markers may not explain all the bone remodeling observed on histological examination. TNF-α activates osteoblasts to induce RANKL for differentiation of osteoclasts [16] or it

directly activates the macrophages, which are the antecedents of osteoclasts, and finally guides the differentiation of macrophages into osteoclasts [17, 18]. Cell proliferation in TIS of bone marrow was apparently induced with infliximab therapy. TNF-α may be needed to stimulate bone remodeling such as in fracture healing. Furthermore, in this study although TNF therapy was administered, expressions of both TNF-α and IL-6 were detected. It is possible that these cytokines may be needed or important for bone formation in patients undergoing treatment with infliximab during bone healing. These findings indicated that infliximab activated bone metabolism and that new bone tissues were confirmed in the bone marrow by histology.

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A Novel Method to Express SNP-Based Genetic Heterogeneity, Ψ, and Its Use to Measure Linkage Disequilibrium for Multiple SNPs, D_g, and to Estimate Absolute Maximum of Haplotype Frequency

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Single nucleotide polymorphisms (SNPs) are important markers to investigate genetic heterogeneity of population and to perform linkage disequilibrium (LD) mapping. We propose a new method, Ψ, to express frequency of 2^{N_s} haplotypes for N_s di-allelic SNPs. Using the new expression of haplotype frequency, we propose a novel measure of LD, D_g, not only for SNP pairs but also for multiple markers. The values of D_g for SNP pairs were revealed to be similar to values of conventional pairwise LD indices, D' and r^2, and it was revealed that D_g quantitated components of LD that were not measured by conventional LD indices for SNP pairs. Also we propose a distinct method, D_g-based absolute estimation, to infer the absolute maximum estimates of haplotype frequency. The result of the D_g-based absolute estimation of haplotype frequency for SNP pairs were compared with the conventional expectation-maximization (EM) algorithm and reported that the new method gave better inference than the EM algorithm which converged infrequently to a local extreme. *Genet. Epidemiol.* 31:709–726, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

DNA molecules are chemical compounds that carry genetic information in living organisms. As carriers of genetic information, they have to be homogeneous enough to maintain identity of species and successful reproduction (speciation hypothesis) [Rieseberg 2001; Noor et al., 2001; Navarro and Barton, 2003; Rieseberg and Livingstone, 2003]. On the contrary, they carry heterogeneity that is believed to give benefit to species for survival in ever-changing environments (adaptation) [Hartl and Clark, 1997a]. Heterogeneity of DNA sequence in a population is balanced between pressures in the two directions, toward the homogeneity or clonality and toward the heterogeneity or randomness.

To clarify the heterogeneity of DNA sequence population, let us assume two extreme populations. In one population, all the DNA molecules are completely identical, in other words, a clonal population. In the other one, any nucleotide in the DNA molecules is randomly selected. In this random population, any site in the DNA molecules is polymorphic, and no association exists between sites or between sets of sites. We define the randomness of polymorphism of individual sites as "allele frequency-randomness" and the randomness between sites or between sets of sites as "inter-site randomness". Because only a small fraction of nucleotide sites in a species is polymorphic, the inter-site randomness is observed only among the polymorphic sites. The pressures toward the clonality of individual sites include selection by favorable phenotypes, unsuccessful reproduction through excessive discrepancy between sequences from gametes and genetic drift due to finite effective population size with unbalanced transmission. On the contrary, mutation and recombination of the genetic material to descendent increase the allele frequency randomness and the inter-site randomness [Hartl and Clark, 1997b].

Since the genome sequences from multiple organisms were determined, one of major research targets has been the investigation of intraspecies variations [Collins et al., 2003]. As reported in *Drosophila*, recombination rate seemed correlated with the presence of nucleotide polymorphisms [Aquadro et al., 1994]. Therefore the allele frequency-randomness of individual nucleotides and the inter-site randomness should be quantitated together. Unfortunately nucleotide diversity, the most popular parameter to quantify heterozygosity at the nucleotide level does not take into account the LD [Hartl and Clark, 1997c]. On the other hand, LD indices, such as D' and r^2 as well as multiallelic D' quantitate only allelic inter-site dependency [Devlin and Risch, 1995; Zapata, 2000].

Because single nucleotide polymorphisms (SNPs) are the most common variations [Kidd et al., 2004] and the LD mapping using SNPs is a very promising method to investigate genetic background of various phenotypes, [Morton, 2005], we propose a new method, Ψ, to express the allele frequency-randomness of individual sites and the intersite randomness for diallelic markers together by a uniform expression. The introduction of Ψ enables us to propose D_g as a novel measure of LD for multiple SNPs, which will be beneficial for genetic studies because only limited measures of LD for multiple loci are currently available, such as ε [Nothnagel et al., 2002], are now limited.

Also using Ψ, we propose a new method to infer the absolute maximum estimate of haplotype frequency. Although the conventional expectation-maximization (EM) algorithm is known to give a local maximum periodically [Nin, 2004], Ψ-based method enables us to overcome the shortcoming and to infer the global maximum of haplotype frequency for SNP pairs, we compared both methods and verified that the conventional EM scarcely converges to a local maximum.

INTRODUCTORY EXAMPLES

Before we give generalized expressions of Ψ and D_g, some examples are introduced.

INCOMPLETENESS OF PAIRWISE LD INDEX

Assume three SNP sites, $S = \{s_A, s_B, s_C\}$, and their eight haplotypes

$H = \{$"ABC", "ABc", "AbC", "Abc", "aBC", "aBc", "abC", "abc"$\}$.

When their haplotype frequencies are

$$F_1 = \{f_{ABC}, f_{ABc}, f_{AbC}, f_{Abc}, f_{aBC}, f_{aBc}, f_{abC}, f_{abc}\}$$
$$= \{0.125, 0.125, 0.125, 0.125, 0.125, 0.125, 0.125, 0.125\}$$

or

$$F_2 = \{0.25, 0, 0, 0.25, 0, 0.25, 0.25, 0\}$$

pairwise r^2's [Devlin and Risch, 1995] of three SNP pairs for both F_1 and F_2 are 0, because frequencies of four haplotypes for each pair are $\{0.25, 0.25, 0.25, 0.25\}$ for both cases (See Columns 1 "$F1$" and 2 "$F2$" in Table I). Although the two cases are apparently different in terms of LD, their pairwise LD index values are the same. This difference implicates that there are components of LD which three pairwise LD indices can not describe. In this article, we propose a LD measure D_g to quantitate all LD components.

CALCULATION OF Ψ AND D_g

Ψ is a novel system to express haplotype frequencies and D_g is a new measure of LD for multiple sites. From haplotype frequencies, Ψ is calculated initially, and D_g is deduced from Ψ.

Transformation of F_i to Ψ and Ψ plots. $\Psi = \{\psi_{subset}\}$ for SNP trio S has eight elements, each of which corresponds to a subset of

$$S : \{\{\phi\}(\text{empty}), \{s_A\}, \{s_B\}, \{s_C\}, \{s_A, s_B\}, \{s_A, s_C\},$$
$$\{s_B, s_C\}, \{s_A, s_B, s_C\}\}.$$

The Ψ elements for each subset are defined with frequency of their alleles (f_x: frequency of haplotype or allele "x") as below:

$$\psi_\phi = 1,$$
$$\psi_{s_A} = f_A - f_a,$$
$$\psi_{s_B} = f_B - f_b,$$
$$\psi_{s_C} = f_C - f_c,$$
$$\psi_{s_A,s_B} = f_{AB} - f_{Ab} - f_{aB} + f_{ab},$$
$$\psi_{s_A,s_C} = f_{AC} - f_{Ac} - f_{aC} + f_{ac},$$
$$\psi_{s_B,s_C} = f_{BC} - f_{Bc} - f_{bC} + f_{bc},$$
$$\psi_{s_A,s_B,s_C} = f_{ABC} - f_{ABc} - f_{AbC} + f_{Abc} - f_{aBC} + f_{aBc} + f_{abC} - f_{abc}.$$

Table I gives frequencies of three sites, three site-pairs and trio as well as the corresponding Ψs. For the case of F_1, $\Psi = \{1, 0, 0, 0, 0, 0, 0, 0\}$ and for F_2, $\Psi = \{1, 0, 0, 0, 0, 0, 0, 1\}$. The first seven elements of Ψ for both cases are the same and the last element, ψ_{s_A,s_B,s_C}, represents the difference between the two.

It is noticed that there is a rule of alternation of positive/negative signs in the expression of Ψ's with frequency of haplotypes, implicating that the expression will be generalized for more nucleotide sites as shown later in this article. Also it is noticed that the introduced variables are in the hierarchic and nested structure. In other words, the elements of Ψ for three sites are consisted of the elements of Ψ's for subsets of sites with an additional component specific to the trio. The nested structure is visualized as Ψ plots in Figure 1(a) with detailed description of its structure. The Ψ plots tell that the difference between F_1 and F_2 is located into the row on the bottom, that stands for the trio. Ψ for the trio contains Ψ for SNP pairs. The four circles in the upper left of Ψ plots, corresponding to $\psi_\phi, \psi_{s_A}, \psi_{s_B}$ and ψ_{s_A,s_B}, create a rhombus that is Ψ plot for the site-pair. Two other rhombuses for site-pairs, $\{s_A, s_C\}$ and $\{s_B, s_C\}$ exist in the Ψ plot of the trio as well. All circles except for ψ_{s_A,s_B,s_C} are a part of at least one of three rhombuses for site-pairs. Figure 1(b) shows structure of Ψ plots of one to seven sites. The Ψ plot of n sites contains Ψ plots of $n-1$ sites in its inside. All the elements in the Ψ plot of n sites except one circle at the bottom are a part of at least one of Ψ's of $n-1$ sites as shown in Figure 1(b).

Calculation of D_g from Ψ and D_g plots. D_g quantitates LD for division patterns of multiple SNPs. For example, a division, $(s_A, s_B, s_C) \rightarrow \{(s_A, s_B), (s_C)\}$ means a division of three SNPs into a SNP pair (s_A, s_B)

TABLE I. Haplotype frequencies and their ψ and D_g

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Column ID	F1	F2	Clone1	Clone2	1 SNP	2 SNPs in absolute LD	2 SNPs in LE	2 SNPs in partial LD	3 SNPs in absolute LD-1	3 SNPs in absolute LD-2	3 SNPs in LE	3 SNPs in partial LD-1	3 SNPs in partial LD-2	3 SNPs in partial LD-3
f_{ABC}	0.125	0.25	1	0	0.6	0.6	0.42	0.6	0.5	0.6	0.336	0.3	0.3	0.3
f_{ABc}	0.125	0	0	1	0	0	0	0	0	0	0.084	0	0	0
f_{AbC}	0.125	0	0	0	0	0	0.18	0.3	0	0	0.144	0	0	0
f_{Abc}	0.125	0.25	0	0	0	0	0	0	0	0	0.036	0.2	0.2	0.3
f_{aBC}	0.125	0	0	0	0.4	0	0.28	0	0	0	0.224	0.3	0.2	0.2
f_{aBc}	0.125	0.25	0	0	0	0	0	0	0	0	0.056	0	0	0
f_{abC}	0.125	0.25	0	0	0	0.4	0.12	0.1	0	0	0.096	0	0	0
f_{abc}	0.125	0	0	0	0	0	0	0	0.5	0.4	0.024	0.2	0.3	0.2
f_A	0.5	0.5	1	1	0.6	0.6	0.6	0.9	0.5	0.6	0.6	0.5	0.5	0.6
f_a	0.5	0.5	0	0	0.4	0.4	0.4	0.1	0.5	0.4	0.4	0.5	0.5	0.4
f_B	0.5	0.5	1	1	1	0.6	0.7	0.6	0.5	0.6	0.7	0.6	0.5	0.5
f_b	0.5	0.5	0	0	0	0.4	0.3	0.4	0.5	0.4	0.3	0.4	0.5	0.5
f_C	0.5	0.5	1	0	1	1	1	1	0.5	0.6	0.8	0.6	0.5	0.5
f_c	0.5	0.5	0	1	0	0	0	0	0.5	0.4	0.2	0.4	0.5	0.5
f_{AB}	0.25	0.25	1	1	0.6	0.6	0.42	0.6	0.5	0.6	0.42	0.3	0.3	0.3
f_{Ab}	0.25	0.25	0	0	0	0	0.18	0.3	0	0	0.18	0.2	0.2	0.3
f_{aB}	0.25	0.25	0	0	0.4	0	0.28	0	0	0	0.28	0.3	0.2	0.2
f_{ab}	0.25	0.25	0	0	0	0.4	0.12	0.1	0.5	0.4	0.12	0.2	0.3	0.2
f_{AC}	0.25	0.25	1	0	0.6	0.6	0.6	0.9	0.5	0.6	0.48	0.3	0.3	0.3
f_{Ac}	0.25	0.25	0	1	0	0	0	0	0	0	0.12	0.2	0.2	0.3
f_{aC}	0.25	0.25	0	0	0.4	0.4	0.4	0.1	0	0	0.32	0.3	0.2	0.2
f_{ac}	0.25	0.25	0	0	0	0	0	0	0.5	0.4	0.08	0.2	0.3	0.2
f_{BC}	0.25	0.25	1	0	1	0.6	0.7	0.6	0.5	0.6	0.56	0.6	0.5	0.5
f_{Bc}	0.25	0.25	0	1	0	0	0	0	0	0	0.14	0	0	0
f_{bC}	0.25	0.25	0	0	0	0.4	0.3	0.4	0	0	0.24	0	0	0
f_{bc}	0.25	0.25	0	0	0	0	0	0	0.5	0.4	0.06	0.4	0.5	0.5
ψ_Φ	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ψ_{s_A}	0	0	1	1	0.2	0.2	0.2	0.8	0	0.2	0.2	0	0	0.2
ψ_{s_B}	0	0	1	1	1	0.2	0.4	0.2	0	0.2	0.4	0.2	0	0
ψ_{s_C}	0	0	1	−1	1	1	1	1	0	0.2	0.6	0.2	0	0
ψ_{s_A,s_B}	0	0	1	1	0.2	1	0.08	0.4	1	1	0.08	0	0.2	0
ψ_{s_A,s_C}	0	0	1	−1	0.2	0.2	0.2	0.8	1	1	0.12	0	0.2	0
ψ_{s_B,s_C}	0	0	1	−1	1	0.2	0.4	0.2	1	1	0.24	1	1	1
ψ_{s_A,s_B,s_C}	0	1	1	−1	0.2	1	0.08	0.4	0	0.2	0.048	0	0	0.2

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TABLE I. Continued.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Column ID	F1	F2	Clone1	Clone2	1 SNP	2 SNPs in absolute LD	2 SNPs in LE	2 SNPs in partial LD	3 SNPs in absolute LD-1	3 SNPs in absolute LD-2	3 SNPs in LE	3 SNPs in partial LD-1	3 SNPs in partial LD-2	3 SNPs in partial LD-3
$d_g((s_A, s_B) \rightarrow \{(s_A), (s_B)\})$	0	0	0	0	0	1	0	0.286	1	1	0	0	0.2	0
$d_g((s_A, s_C) \rightarrow \{(s_A), (s_C)\})$	0	0	0	0	0	0	0	0	1	1	0	0	0.2	0
$d_g((s_B, s_C) \rightarrow \{(s_B), (s_C)\})$	0	0	0	0	0	0	0	0	1	1	0	1	1	1
$d_g((s_A,s_B,s_C) \rightarrow \{(s_A,s_B),(s_C)\})$	0	1	0	0	0	0	0	0	0	0	0	0	0	0.2
$d_g((s_A,s_B,s_C) \rightarrow \{(s_A,s_C),(s_B)\})$	0	1	0	0	0	1	0	0.286	0	0	0	0	0	0.2
$d_g((s_A,s_B,s_C) \rightarrow \{(s_A),(s_B,s_C)\})$	0	1	0	0	0	1	0	0.286	0	0	0	0	0	0
$d_g((s_A,s_B,s_C) \rightarrow \{(s_A),(s_B),(s_C)\})$	0	1	0	0	0	1	0	0.286	0	0.194	0	0	0	0.2

Cells with 1 or −1 in ψ or d_g are shadowed.

and a single SNP s_C. There are seven division patterns for three sites:

$$(s_A, s_B) \rightarrow \{(s_A), (s_B)\},$$
$$(s_A, s_C) \rightarrow \{(s_A), (s_C)\},$$
$$(s_B, s_C) \rightarrow \{(s_B), (s_C)\},$$
$$(s_A, s_B, s_C) \rightarrow \{(s_A, s_B), (s_C)\},$$
$$(s_A, s_B, s_C) \rightarrow \{(s_A, s_C), (s_B)\},$$
$$(s_A, s_B, s_C) \rightarrow \{(s_A), (s_B, s_C)\},$$
$$(s_A, s_B, s_C) \rightarrow \{(s_A), (s_B), (s_C)\}.$$

The first three divides a SNP pairs into two single SNPs. The next three split a SNP trio into a SNP pair and a single SNP. The last divides a SNP trio into three single SNPs.

The elements of $D_g = \{d_g((\text{subset}_i) \rightarrow \{(\text{subset}_{ji}), (\text{subset}_{jz}), ..., (\text{subset}_{jk})\})\}$, correspond to divisions. We define D_g as below. Of note when the denominator of either expression in the parenthesis is zero, take the other value.

$$d_g((s_A,s_B)\rightarrow\{(s_A),(s_B)\})$$
$$=\max\left(\left(1-\frac{\psi_{s_A,s_B}+1}{\psi_{s_A}\times\psi_{s_B}+1}\right),\left(1-\frac{\psi_{s_A,s_B}-1}{\psi_{s_A}\times\psi_{s_B}-1}\right)\right),$$
$$d_g((s_A,s_C)\rightarrow\{(s_A),(s_C)\})$$
$$=\max\left(\left(1-\frac{\psi_{s_A,s_C}+1}{\psi_{s_A}\times\psi_{s_C}+1}\right),\left(1-\frac{\psi_{s_A,s_C}-1}{\psi_{s_A}\times\psi_{s_C}-1}\right)\right),$$
$$d_g((s_B,s_C)\rightarrow\{(s_B),(s_C)\})$$
$$=\max\left(\left(1-\frac{\psi_{s_B,s_C}+1}{\psi_{s_B}\times\psi_{s_C}+1}\right),\left(1-\frac{\psi_{s_B,s_C}-1}{\psi_{s_B}\times\psi_{s_C}-1}\right)\right),$$
$$d_g((s_A,s_B,s_C)\rightarrow\{(s_A,s_B),(s_C)\})$$
$$=\max\left(\left(1-\frac{\psi_{s_A,s_B,s_C}+1}{\psi_{s_A,s_B}\times\psi_{s_C}+1}\right),\left(1-\frac{\psi_{s_A,s_B,s_C}-1}{\psi_{s_A,s_B}\times\psi_{s_C}-1}\right)\right),$$
$$d_g((s_A,s_B,s_C)\rightarrow\{(s_A,s_C),(s_B)\})$$
$$=\max\left(\left(1-\frac{\psi_{s_A,s_B,s_C}+1}{\psi_{s_A,s_C}\times\psi_{s_B}+1}\right),\left(1-\frac{\psi_{s_A,s_B,s_C}-1}{\psi_{s_A,s_C}\times\psi_{s_B}-1}\right)\right),$$
$$d_g((s_A,s_B,s_C)\rightarrow\{(s_B,s_C),(s_A)\})$$
$$=\max\left(\left(1-\frac{\psi_{s_A,s_B,s_C}+1}{\psi_{s_B,s_C}\times\psi_{s_A}+1}\right),\left(1-\frac{\psi_{s_A,s_B,s_C}-1}{\psi_{s_B,s_C}\times\psi_{s_A}-1}\right)\right),$$
$$d_g((s_A,s_B,s_C)\rightarrow\{(s_A),(s_B),(s_C)\})$$
$$=\max\left(\left(1-\frac{\psi_{s_A,s_B,s_C}+1}{\psi_{s_A}\times\psi_{s_B}\times\psi_{s_C}+1}\right),\right.$$
$$\left.\left(1-\frac{\psi_{s_A,s_B,s_C}-1}{\psi_{s_A}\times\psi_{s_B}\times\psi_{s_C}-1}\right)\right).$$