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ORIGINAL PAPER

Crossing over analysis at pachytene in man

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The distribution of anti-MLH1 (MutL homologue 1) antibody labelling was studied in human prophase meiocytes. A labelling pattern consisting of distinct foci, always associated with the synaptonemal complex (SC) and never in closely juxtaposed pairs, was observed. Comparison of the number and general positions of autosomal foci with previous studies of the number and positions of autosomal chiasmata indicates that the anti-MLH1 antibody marks sites of crossing over in human pachytene spermatocytes. A mean number of 50.9 autosomal foci was observed from 46 human pachytene spermatocytes corresponding to a genetic length of 2545 cM. Division of these spermatocytes into sub-stages revealed that the number of foci remains stable throughout pachytene. A focus was found on the XY bivalent in 56.5% of the nuclei. The presence or absence of foci from the XY bivalent could not be correlated to pachytene sub-stage.

Keywords: Crossing over; genetic length; human; meiosis; MLH-1; oocyte; pachytene; synaptonemal complex; spermatocyte

Introduction

Meiosis is the process whereby the genetic content of cells is reduced from the somatic to the gametic content, in order to maintain a fixed volume of genetic material upon fusion of gametes. Crucial to this process is the co-localisation and intimate physical coupling of homologous chromosomes during the first meiotic division in order to make the even distribution of genetic material between daughter cells manageable. Intimate coupling of chromosomes occurs during zygotene when homologous chromosomes synapse and is maintained throughout pachytene by the ribbon-like synaptonemal complex (SC).[1] Upon relaxation of synapsis and dissolution of the synaptonemal complex during diplotene, coupling is maintained by chiasmata, reviewed by Carpenter,[2] cytologically detectable evidence of crossover events that are initiated prior to synapsis.[3–5] Electron microscope (EM) studies of pachytene meiocytes have revealed the presence of SC associated granules whose positions show good correspondence with sites of chiasmata, known as late recombination nodules (late RNs).[6–9] Late recombination nodules are with traditional staining techniques both beyond the resolution of the light microscope and apparently ephemeral structures with no reports of the observation of a full complement in humans.[10]

In a recent immunofluorescence study of the distribution of the DNA mis-match repair protein M1h1 in microspread mouse prophase meiocytes,[11] it was concluded that this protein localises to sites of crossing over as a possible component of late RNs. This is not only of fundamental importance in the attempt to unravel the molecular events of meiotic recombination

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in higher eukaryotes, but as a 'spin off' provides a potentially extremely useful cytogenetic tool for the identification of sites of crossing over in microspread pachytene nuclei at the level of the light microscope. The traditional cytological means of collecting data on the number and positions of crossover events has been dependent upon the study of chiasmata at either diplotene, when they are difficult to distinguish from twists, or diakinesis/metaphase I when chromosome morphology is not finely detailed and the highly contracted state of the chromosomes makes discrimination between terminal and sub-terminal chiasmata troublesome.[9,12,13] The need to recover large numbers of high quality diakinesis/metaphase I nuclei from air dried preparations has been a constant technical challenge, in particular from human females.[12] Numerous pachytene nuclei are easily recoverable from microspread preparations and their chromosomes are highly extended structures[14] against which crossover events could be mapped.

In this study the distribution of MLH1 in human spermatocytes was examined in order to assess the applicability of the conclusions of Baker *et al*[11] and the usefulness of anti-MLH1 labelling as a cytogenetic tool in humans for the identification of meiotic crossover points. A preliminary study of MLH1 distribution in oocytes was also made.

Materials and Methods

Human male-microspreading and immunocytology

A small quantity of seminiferous tubules was recovered from the testis of a 45-year-old human male of proven fertility via needle biopsy under local anaesthesia.

Testicular tissue was macerated in Earls Balanced Salt Solution (EBSS) to produce a thin cell suspension, and microspread according to the method described[15] with slight modifications. One drop of cell suspension was mixed with one drop of 0.003% Lipsol solution on a clean microscope slide and allowed to stand for 10 min. Six drops of 2% ultrapure formaldehyde (TAAB Laboratory Equipment, Aldermaston, UK) containing 0.02% SDS pH 8.4 (sodium borate) were added for 10 min, after which the slides were briefly dipped in distilled water and air dried. Slides were then blocked in PBT (PBS, 0.15% BSA, 0.1% Tween 20) for approximately 30 min. A mixture of serum A1 (knuf) and anti-MLH1 antibody (Pharmingen, San Diego, California) was applied at concentrations of 1:1000, and 1:500, respectively, in PBT overnight at room temperature. Serum A1 (knuf), a gift of Christa Heyting, University of Wageningen, The Netherlands, was raised against rat synaptonemal complexes (SCs) in rabbits and predominantly recognises SCP3, a lateral element component.[16] The anti-MLH1 antibody is a mouse monoclonal antibody. Slides were then given three 5 min washes in PBT and a secondary TRITC conjugated goat anti-rabbit IgG antibody (Sigma, Poole, UK) combined with a FITC conjugated goat anti-mouse IgG antibody (Sigma, Poole, UK) was applied, each at a concentration of 1:500, in PBT for 4 h at room temperature. After three 5 min washes in PBT followed by a brief rinse in distilled water, air-dried slides were stored at –70°C. Upon removal from the freezer slides were stained in DAPI (0.2 μg/ml) in 2 × SSC, briefly rinsed in water and vectorshield (Vector Laboratories, Peterborough, UK) applied beneath a coverslip. Slides were studied with a Zeiss axioskop epifluorescence microscope fitted with a Pinkel number 1 filter set and a cooled charged coupled device (CCD) (Photometrics, Münich, Germany). Images in which anti-MLH1 antibody foci were visible were captured on a Power Macintosh 8100/80 with Smartcapture software (Digital Scientific, Cambridge, UK).

Human female-microspreading and immunocytology

A normal human female foetus (16 weeks gestation) was delivered after cervigen induced termination (conducted for social reasons) in the early evening and ovaries removed the next morning. Ovaries were kept in EBSS culture medium at room temperature for approximately 5 h prior to microspreading. After chopping with springbow scissors in EBSS, the pieces were teased apart with scalpel blades to produce a cell suspension. A small portion of the cell suspension was used during this experiment, with the majority divided amongst other experiments. One drop of suspension was mixed with five drops of a 0.3% solution of lipsol on a clean microscope slide and allowed to stand for 30 min; 10 drops of 1% ultrapure formaldehyde (TAAB Laboratory Equipment, Aldermaston, UK), 0.04% SDS were added for 20 min after which the slides were briefly dipped in distilled water and air dried.

Slides were blocked, labelled and studied as described for human males.

Results

Male-number of foci

Anti-MLH1 antibody labelling was visible as distinct foci on synaptonemal complexes in pachytene nuclei from stages 1 to 5 according to the classification of Solari.[17] Nuclei in which at least one focus per autosomal bivalent was visible were considered fully labelled, whilst nuclei that contained some foci free autosomal bivalents were considered partially labelled; 46 fully labelled nuclei were observed, in which the mean number of autosomal foci was 50.9 (n = 46, stdev = 4.4, range 41–59). A focus was seen in the pairing segment of the XY bivalent in 26 nuclei (56.5%). Nuclei were divided into early pachytene (stages 1 and 2, X and Y axial elements intact[17]) and late pachytene (stages 3 to 5, X and Y axial elements shredded.[17]) Early pachytene nuclei contained a mean number of autosomal foci of 49.9 (n = 17, stdev = 4.7, range = 41–58), whilst late pachytene nuclei contained a mean of 51.6 (n = 29, stdev = 4.2, range = 43–59), see

Table 1. A focus was seen associated with the XY bivalent in 58.8% of early and 55.2% of late nuclei (Table 1). Further division of nuclei into pachytene sub-stages 1–5 (stages 4 and 5 could not be discriminated and were therefore pooled) did not reveal a divergence from a mean of around 50 autosomal foci for any stage, (Table 2). Five partially anti-MLH1 antibody labelled nuclei were observed, two were considered stage 1 (eg Figure 1a), one was considered stage 2, one was considered stage 5 (Figure 2b) and one was a diplotene nucleus. Inclusion of these partially labelled nuclei in the mean autosomal foci calculation has a significant effect on only the mean of stage 1 nuclei, for which there were only 3 fully labelled nuclei, (Table 3).

Male-positions of foci

When more than one focus was found per bivalent the foci were separated by a distance no smaller than the approximate length of the smallest bivalent (chromosome 21) in the nucleus (Figure 1b–1d, Figure 2a). In only a single bivalent was an exception to this generalisation observed (Figure 2c, green arrow). Near terminal positions were favoured by MLH1 foci (Figure 1b–1d, Figure 2a–2c). In the single diplotene nucleus observed a few anti-MLH1 antibody foci remained, often in places where desynapsing bivalents remained associated (data not shown). Two foci were visible between pairs of de-synapsing axial elements that converged but did not actually meet (data not shown).

Female-number and positions of foci

Obtaining human foetal ovaries suitable for study has obvious logistical hurdles which have restricted our studies to only 15 nuclei from a 16 weeks' gestation foetus: 4 nuclei were zygotene (3 late zygotene) and were unlabelled; 6 nuclei were pachytene of which 1 was unlabelled, 1 contained a few faint foci, and 3 contained multiple distinct foci (see Figure 2d and Figure 2e); 6 nuclei were diplotene, 3 of which contained foci; the remaining 3 were unlabelled. The 3 well labelled pachytene nuclei contained 81, 101, and 104 foci, with a mean number of 95 (n = 3, stdev = 12.3, range = 81–104). Terminal locations of MLH1 foci were apparently less favoured in human females than males. In common with human males, foci were not seen closely juxtaposed but were separated by a minimum distance.

Table 1 Division of early and late pachytene nuclei

stages	*1+2*	*3, 4+5*
mean autosomal foci	49.9	51.6
range	41–58	43–59
stdev	4.7	4.2
XY with foci	58.8%	55.2%
n	17	29

Discussion

The use of an anti-MLH1 monoclonal antibody has produced a labelling pattern consisting of discrete foci in both human male and female pachytene nuclei, as has previously been reported for mice.[11] Baker *et al*[11] concluded that Mlh1 foci marked the sites of crossing over, reflecting a role, at present unclear, in this process (reviewed by Arnheim and Shibata).[18] Comparison of the mean number of autosomal anti-MLH1 antibody foci in human spermatocytes of 50.9 (n = 46, stdev = 4.4, range 41–59) with the mean number of autosomal chiasmata measured in air-dried nuclei during light microscope studies reveals that the means show a remarkable coincidence (see Table 4) with only the means of Fang and Jagiello,[24] who uniquely studied diplotene nuclei (other workers studied diakinesis nuclei), and McDermott[20] deviating from the consensus of around 51. Hultén[21] noted distal and terminal chiasmata were more common than medial ones, a finding corroborated by linkage-based genetic maps, which suggest high levels of genetic recombination in telomeric regions,[25] in particular in males[26–28] which is in keeping with the observed distribution of MLH1 foci.

Table 2 Division of fully labelled nuclei into pachytene sub-stages

stage	*1 (eg Fig. 1b)*	*2 (eg Fig. 1c)*	*3 (eg Fig. 1d)*	*4–5 (eg Fig. 2a)*
mean autosomal foci	48.7	50.1	51.5	51.6
range	45–51	41–58	43–59	43–59
stdev	3.2	5.2	5.58	3.9
XY with foci	66.7%	57.1%	75%	52%
n	3	14	4	25

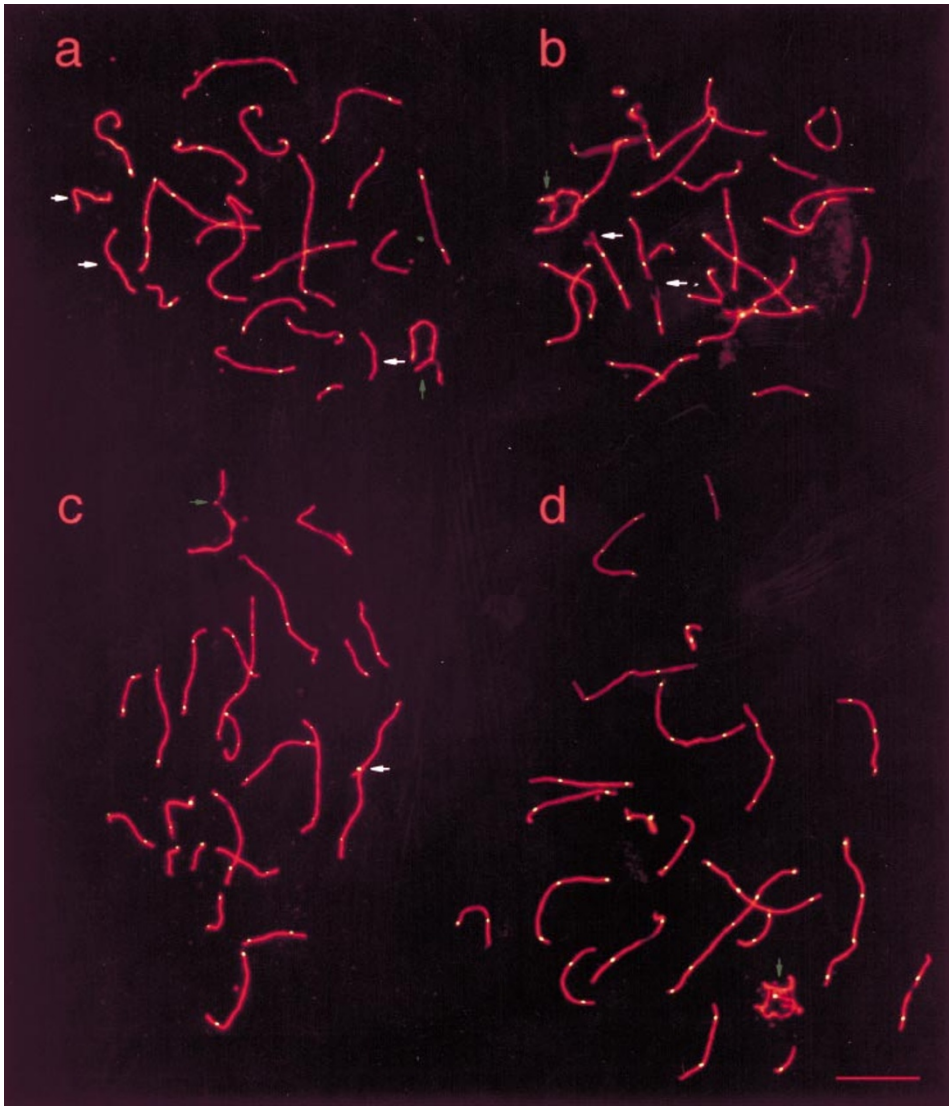

Figure 1 *Microspread human spermatocytes labelled with anti-SCP 3 antibody (red) and anti-MLH1 monoclonal antibody (yellow). (***a***) Early pachytene nucleus (stage 1) with a partial complement of anti-MLH1 antibody foci. Note the presence of several bivalent that are free of anti-MLH1 antibody labelling (white arrows). Note also the large region of synapsis of the XY bivalent (green arrow). (***b***) Early pachytene nucleus (stage 1–2) with a full complement of anti-MLH1 antibody foci. No foci free bivalents can be seen. Note the large region of XY synapsis (green arrow) and some autosomal regions that remain asynapsed (white arrows). (***c***) Pachytene nucleus (stage 2) containing a full complement of anti-MLH1 foci. The XY bivalent of this nucleus has undergone almost complete desynapsis, with only a remnant of synapsis remaining in which an anti-MLH1 focus can be seen (green arrow). (***d***) Mid-pachytene nucleus in which the axial elements of the XY have started to split and anastamose. Note the presence of an anti-MLH1 focus on the XY bivalent (green arrow). Scale bar–10 μm.*

Studies of chiasma numbers per bivalent of air-dried nuclei suggested that chromosome 21 receives a mean 1.05 chiasma (52.5 cM) per bivalent.[21] Recent application of the mathematical methods of Weinstein[29] to CEPH data to estimate chiasma distributions to human chromosome 21 estimated 1.03 exchange events (51.5 cM) per bivalent,[30] the smallest bivalent in each nucleus observed during our study was labelled with only a single anti-MLH1 focus (50 cM).

Multiple MLH1 foci were separated by a distance approximately equivalent to the length of chromosome 21, similar to the known distribution of chiasmata,[21] the result of positive interference. This study therefore confirms the conclusion of Baker *et al*[11] that foci of anti-

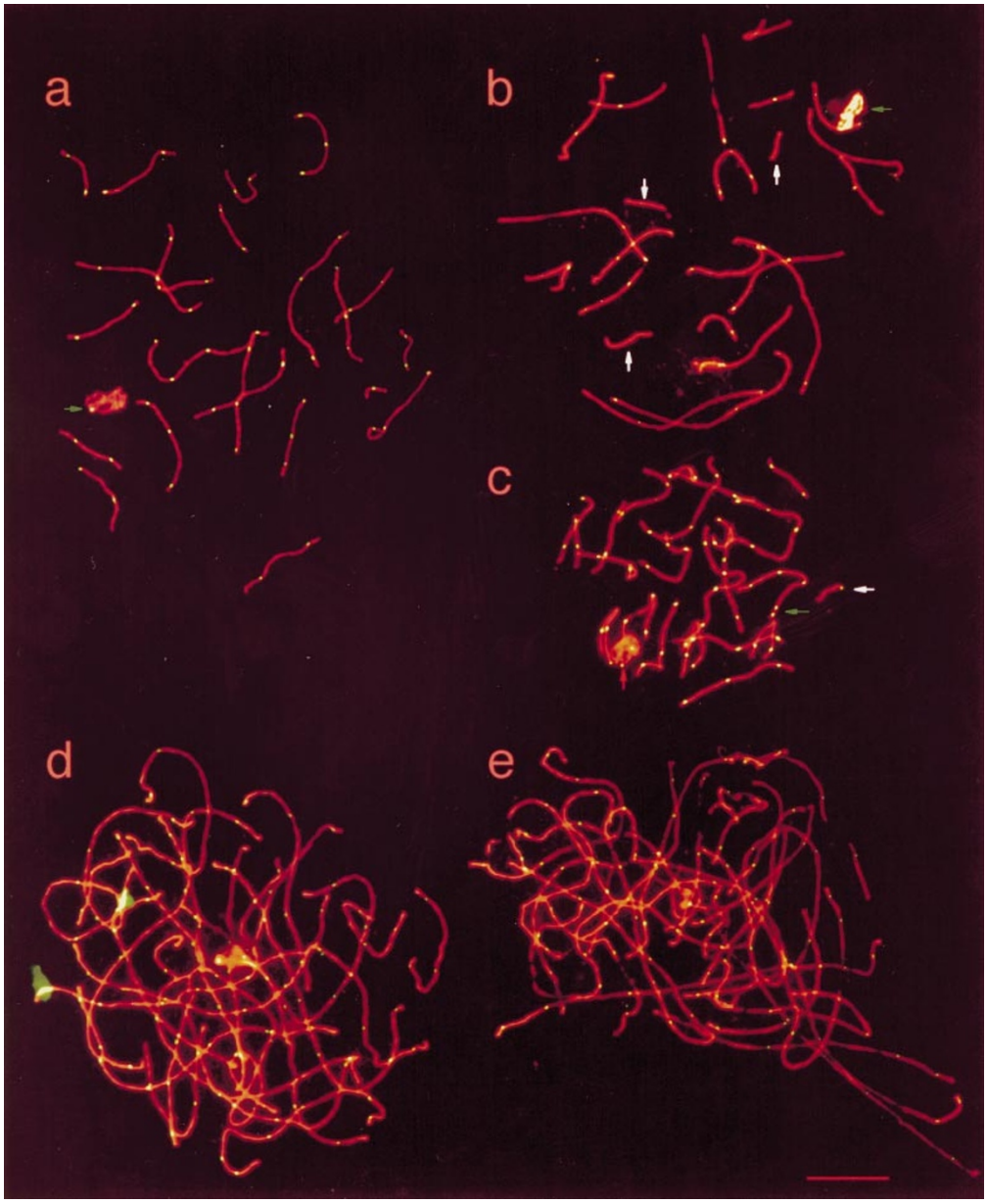

Figure 2a–c *Microspread human spermatocytes labelled with anti-SCP 3 antibody (red) and anti-MLH1 monoclonal antibody (yellow). (***a***) Late pachytene nucleus (stage 4–5) in which the XY bivalent has become highly shredded and anastamosed, but retains an MLH1 focus (green arrow). This nucleus retains a full complement of MLH1 foci. (***b***) Late pachytene nucleus (stage 4–5) containing a shredded and anastamosed XY bivalent. This nucleus contains a partial complement of MLH1 foci with some bivalents focus free (white arrows). (***c***) Late pachytene nucleus (stage 4–5). This is an exceptional nucleus in which two foci were closely juxtaposed (green arrow) by a distance substantially smaller than the length of the bivalent 21 (white arrow).*
Figure 2d–e *Microspread human pachytene oocytes labelled with anti-SCP 3 antibody (red) and anti-MLH1 monoclonal antibody (yellow). There are no closely juxtaposed foci. Note the presence of 104(d) and 100(e) foci. Scale bar–10 μm.*

MLH1 antibody labelling mark sites of crossing over that would be resolved as chiasmata by metaphase I.

During our study 50.9 reciprocal recombination events observed as MLH1 foci makes the genetic length in human males 2545 cM, which compares to 2729.7 cM from linkage data,[25] figures which show a remarkable degree of correspondence, especially when one considers that in many organisms genetic mapping frequently results in maps with lengths exceeding those based on chiasma counts.[31] Various sources of error in the production of genetic maps have been proposed to account for such 'map inflation'.[31]

Table 3 Division of all nuclei into pachytene sub-stages (inclusive of partially labelled nuclei)

stage	*1 (eg Fig. 1b)*	*2 (eg Fig. 1c)*	*3 (eg Fig. 1d)*	*4–5 (eg Fig. 2a)*
mean autosomal foci	41.8	49.1	51.5	50.7
range	29–51	36–58	43–59	28–59
stdev	9.8	6.2	5.58	6
XY with foci	40%	53.3%	75%	50%
n	5	15	4	26

Table 4 Chiasmata numbers recorded in 46, XY males

Reference no.	*No. of subjects*	*No. of cells*	*Mean no. (cM)*	*SD*	*Range*
19	124 (pooled data from 11 studies)	2639	50.6 (2530)	1.6–6.7	33–66
20	21	516	53.7 (2685)		43–62
21	1	41	50.6 (2530)	3.87	43–60
22			51.1 (2555)	3	
23	7	408	51.3 (2566.5)	1.39	49.6–53.7
24	6	91	45.3 (2266.5)	4.52	32–58
MLHI foci (this study)	1	46	50.9 (2545)	4.4	41–59

Clear differences exist between the reported Mlh1 temporal distribution in mouse spermatocytes and that of humans. Baker *et al*[11] report fluctuations in the numbers of Mlh1 foci, with an initial increase by mid-pachytene followed by a gradual decrease. If the mean number of foci is calculated with the inclusion of partially labelled nuclei then the impression may be gained that this is also the situation in humans (see Table 3) with numbers increasing between pachytene stages 1 and 2. However, exclusion of partially labelled pachytene nuclei (of which there were only 4/50) reveals that following the acquisition of a full complement of foci the number present is remarkably stable at around 50 throughout pachytene. The observation of only four partially labelled pachytene nuclei of which three were early suggests the acquisition of foci occurs at pachytene stages 1–2 and is a rapid process, as many more such nuclei would have been observed if the process were protracted. Only 3 fully labelled nuclei were regarded as being in pachytene stage 1, the presence of both partially labelled nuclei and a low number of fully labelled nuclei within this stage adds weight to the conclusion that foci are acquired during pachytene stage 1. Baker *et al*[11] reported the disappearance of anti-Mlh1 antibody foci from mouse spermatocytes by late pachytene, whereas in the human male, the single diplotene nucleus observed during this study contained foci.

The XY bivalent was found to have a clear focus of MLH1 in approximately half the spermatocytes studied (58.8% of early nuclei, 55.2% of late nuclei, see Table 1); division of nuclei into four sub-stages (Table 2) did not reveal a clear trend in the stages at which this focus is present. Baker *et al*[11] on the other hand consistently observed foci on the XY bivalent in early stages and saw no foci in later stages when the XY had desynapsed. Clearly in the human male the XY focus can persist long after the majority of the synaptic region has desynapsed. EM studies indicate that even in very late stages of pachytene when the XY had desynapsed and the axial elements of the sex chromosomes have become shredded and anastomosed a remnant of a pairing segment remains,[32] presumably persistent XY MLH1 foci lie within this remnant (Figure 2a, green arrow). Studies of the segregation of RFLPs within pedigrees indicate that a single obligatory crossover occurs between the XY in human male meiosis.[33] The observation of MLH1 foci on little more than half the observed sex bivalents may be an indication of either the eccentric timing of recombination for this unique bivalent, or alternatively the presence of large numbers of spermatocytes destined for spermatogenic arrest due to their failure to receive an obligatory crossover.[34]

Data regarding the MLH1 distribution during human female meiosis was strictly limited during this study due to difficulty in obtaining a suitable volume of material for study. On the basis of the very limited number of observations there is no evidence of a different temporal distribution to that seen in human males, ie foci acquired during pachytene (Figure 2d and Figure 2e) and persisting until diplotene. The mean number of MLH1 foci of 95 (n = 3, stdev = 12.3,

range = 81–104, 4750 cM) was based on only 3 observations and therefore carries little weight, in particular in view of the fact that Baker *et al*[11] report a rapid loss of foci during early pachytene in female mice. However, the evidence from linkage studies that the genetic length in human females is approximately 60% longer than that of males[25] confirms that many more recombination events should be expected in female meiosis. Cytogenetic data regarding chiasma numbers and distribution has been severely restricted by the technical difficulties involved in scoring chiasmata in cultured oocytes. The published frequencies which range from 38.4 to 48 per nucleus[35–37] have been criticised on the grounds that they are serious underestimates, a result of the great technical difficulties in scoring chiasmata in oocytes cultured *in vitro*.[12] However cytogenetic evidence exists,[12] supported by genetic evidence[25,26] that suggests that terminal chiasmata are less frequent in female meiosis than in male meiosis, which appears to fit with the MLH1 distribution presented here. Clearly many more observations are required in order to accurately elucidate the number of recombination events as well as their temporal and physical distribution in females.

Baker *et al*[11] speculated that Mlh1 may be a component of late recombination nodules (RNs), the cytological manifestations of crossing over identified in pachytene nuclei during EM studies.[6–9] If MLH1 is indeed a component of late RNs, then our study is the first in which a full complement of recombination nodules have been identified in human spermatocytes (reviewed by Ashley).[10] During complex reconstruction of serially sectioned human spermatocytes Holm and Rasmussen[38] failed to find a full set of 50 late RNs in a single nucleus, with a mean of 35 late RNs found in mid-pachytene nuclei. This may have been due to difficulties in identifying RNs in sectioned material, as has been shown in plants.[39] Alternatively, nodules may be highly ephemeral structures in humans. If this is the case then MLH1 foci may not be components of late RNs, but may be left at sites where late RNs have been. In the case of females the mean of 95 MLH1 foci (n = 3) observed during this study exceeds the means of 60, 59 and 46 RNs observed in serially sectioned oocytes at early, mid and late pachytene,[40] again reflecting either the difficulty of staining RNs in the EM or the possibility that RNs are ephemeral structures with MLH1 foci marking sites where RNs have been.

The role of proteins homologous to bacterial mismatch repair proteins at sites of crossing over is unclear. Ross-Macdonald and Roeder[41] suggested that in yeast Msh4 (MutS homologue 4) may recognise a substrate other than mis-matches, such as Holliday junctions, and that the structural perturbation generated may resemble a mis-match. They proposed that its role may be the stabilisation of Holliday junctions, counteracting the forces that act to undo strand exchange intermediates, and/or Holliday junction cleavage, isomerisation or stabilisation of previously isomerised Holliday junctions until resolution. Hunter and Borts[42] speculated that Mlh1 in yeast has a role after Msh4 and Msh5 reinforcing Holliday junction stabilisation and promoting progression to crossover resolution.

Conclusion

A previous study of the distribution of the mis-match repair protein Mlh1 in mouse meiocytes has indicated that the sites at which foci of this protein are localised correspond to sites of crossing over.[11] Our study confirms that the same is true for human spermatocytes. Observation of anti-MLH1 antibody foci will provide a useful cytogenetic tool in the study of crossover events in normal males, infertile males, and structural chromosomal rearrangement carriers. The number and distribution of chiasmata is known to show characteristic patterns in normal males[23,43,44] but both unusual numbers eg[45–47] and an unusual distribution have been observed in infertile males[48] and translocation carriers.[49,50] The use of air-dried diakinesis nuclei in studies of chiasma distribution is restrictive due to the low proportion of such nuclei recovered, and subject to inaccuracy due to difficulties such as distinguishing subterminal and terminal chiasmata.[9,12,13] The use of MLH1 foci on microspread pachytene chromosomes would provide the advantages of high numbers of readily accessible nuclei and highly extended chromosomes. The fine mapping of chiasmata will be possible by combining the immunocytogenetic studies (ie the anti-MLH1 antibody + anti SC antibodies) with FISH techniques for chromosomal identification, the use of this combination of technologies has been demonstrated in both human spermatocytes and oocytes.[51–53] The observation of MLH1 foci within pachytene meiocytes provides a unique and straightforward alternative to chiasma analysis of simultaneously observing the number and positions of total recombination events

within a single nucleus, providing an overview unavailable from linkage data.

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