Study the influence of some *Listeria monocytogenes* antigens on the side effects of Mitomycin C

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Summary

For studying *in vivo* influence of immune responses on side effects of Mitomycin C (MMC),Sixty-five male-white mice were divided to nine groups ;1st group inoculated MMC,I.P,0.4mg/ml,2 doses weekly/4 weeks;2nd to 7th groups immunized S/C with *LM* Ags(25mg/ml) protein concentration 0.4 ml, 2doses/2 weeks intervals;2nd and 3rd groups immunized CF*LM*Ags and CF*LM*Ags-MMC in 3rd group; also 4th and fifth groups but immunized with WS*LMA*gs; 6th and 7th groups inoculated with 0.4 ml Attenuated *LM* (La*LM*Ags), 7th group immunized-MMC treated. Post-immunization, 5 animals sacrificed from 1-7groups and negative control to study the cellular and humoral immunity as well as bone marrow taken to cytogenic examination ,other animals from 1-7 groups and 8th group challenged with 0.4 ml of (Vi*LM*) containing($1x10^9$ CFU/ml) of), the animals of negative group inoculated 0.4 ml of sterile PBS,S/C.Results; showed high mean values of IFN-y and IgG1 in animals immunized with *LM*.Ags, while MMC decreased levels of IFN-y and IgG1and MI%, increased chromosomal aberrations and micronucleus while decreased in immunized also immunized–MMC treatment , immunized animals show increased MI%.The suppurative pathological lesions and suppurative granulomatous lesions were appeared clear in tissue sections of internal organs in animals of positive control,decreased severity in immunized,immunized-MMCtreated especially with La*LM* immunization.

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دراسة تاثير بعض مستضدات جرثومة الليستيريا مونوسايتوجينز على التأثيرات الجانبية للمايتومايسين سي

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الخلاصة

ان الهدف من الدراسة الحالية هو التحري عن تاثير بعض مستضدات جرثومة ال *LM* على التاثيرات الجانبية لعقار Mitomycin C. حيث تم اسخدام تسع مجاميع من ذكور الفئران البيضاء, حقنت المجموعة الاولى MMC داخل الخلب 0.4ملغم/مل جرعتين في الاسبوع /اربعة اسابيع,ومنعت المجاميع الثانية الى السابعة بمستضدات جرثومة *LM*, 0.4 مل,جرعتين الفارق بينهما اسبوعين تحت الجلد,حيث منعت المجموعتين الثانية والثالثة بمستضد الراشح الجرثومي وحقنت المجموعة الثالثة بال MMC ,كما في منعت المجموعة الرابعة والخامسة ولكن بالمستضد الكلي المتكسرلجرثومة *WS*,و المجموعة السادسة والسابعة بجراثيم *LM* الحية المضعفة وحقنت المجموعة السابعة بال MMC, ,بعد 28 يوما من التمنيع تم قتل خمس حيوانات من المجاميع الاولى الى السابعة و مجموعة السيطرة السالبة,لجمع عينات الدم لقياس مستوى IgG1 وال IFN-y بالاضافة الى جمع نماذج نقي العظم,بعد ذلك تم حقن الحيوانات الباقية(5) وحيوانات السيطرة الموجبة ب 0.4 مل من العالق الجرثومي الحاوي على CFU/ml $1x10^9$ من جراثيم *LM* الحية الضارية,وحقنت حيوانات السيطرة السالبة 0.4 مل من المحلول الملحي المعقم تحت الجلد .بينت النتائج بان مستضدات *LM* حفزت مستوى عالي من المناعة الخلوية والخلطية ,قبل وبعد الاصابة بجراثيم *LM* ,بينما سبب MMC انخفاض مستوى المناعة قبل وبعد الاصابة,كما لوحظ انخفاض مستوى الانقسام الخيطي MI% وارتفاع نسبة التشوهات الكروموسومية وتكوين النويات الصغيرة في الحيوانات المعاملة بال MMC ,بينما قلت نسبة التشوهات الكروموسومية مع ارتفاع نسبة ال MI% في الحيوانات الممنعة والمعالجة بال MMC .كما بينت الدراسة وجود تغيرات مرضية قيحية واو رام حبيبية قيحية في الاعضاء الداخلية لحيوانات السيطرة الموجبة, كانت التغيرات المرضية اقل شدة في الحيوانات الممنعة والممنعة والمعاملة بال MMC بعد الاصابة وخاصة في الحيوانات الممنعة بجراثيم *LM* المضعفة.

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Introduction

Mitomycin C is a cytotoxic drug in clinical chemotherapy regiments for the treatment of various carcinomas(1,2,3,4) for a more than 30 years, it is cytotoxicity due primarily to the formation of DNA adducts-in particular; DNA interstrand cross-link (ICL) (5). It is also used in combination with other antitumour agents in palliative treatment of many advanced cancers or cancers that have become resistant to other (6).

The cytogenic studies revealed that MMC frequently produce chromatid exchanges in human cells, particularly human chromosomes (1, 9 and 16) are frequently involved in so called quadriradicals (7). Very few workers have studied the influences of stimulated immune system against the cytogenetic effects of MMC on bone marrow cells of mice, hence, the present study was undertaken to determine the influences of some *Listeria monocytogenes* antigens on mitotic activity and chromosomal aberrations in mice immunized-treated MMC and study the pathological changes.

Experimental design: Sixty-five (n=65) of white male mice aged (7-8 weeks), divided randomly into seven groups and treated as following:

1st group: (n=10) inoculated with 0.4ml (0.4mg/ml) MMC, I.P., 2 doses weekly /4weeks. **Snd group**: (n=10), immunized with 0.4ml of Culture Filtrate *Listeria monocytogenes* antigens (CF*LM*Ags) (25 mg/ml), S/C, 2 doses, 2 weeks intervals.**3rd group**: (n=10) immunized as in 2nd group, at the same time inoculated with MMC as in the 1st group. **4th group**: (n=10), immunized as the 2nd group but with Whole-Sonicated *LM*.Ags (WS*LM*.Ags). **5th group**: (n=10) immunized as in 4th group and treated with MMC as the in 1st group. **6th group:** (n=5) as positive control. **7th group:** (n=10) as negative control.

At day 28 post-immunization, skin test was done and five animals from 1st, 2nd, 3rd, 4th, 5th and 7th groups were sacrificed and collect blood to determined IFN-y and IgG$_1$ concentrations and bone marrow for cytogenic analysis (8), the remaining five animals as well as positive control were challenged with 0.4ml (1x10^9 CFU/ml) of (Vi*LM*) and negative group inoculated S/C with 0.4ml of sterile PBS. All the animals were sacrificed and pieces of internal organs were fixed in 10% neutral buffer formalin for 72 hours for histopathological examination according to (9).

Materials and Methods

Mitomycin-C (2mg) was obtained from Sigma-Aldach Company. Chromosome aberration assay according to (10) and Micronucleus test according to the procedure of (11).Preparation of bone marrow cells was carried out according to the procedure produced by (12). Slide Preparation and Staining; according to the procedure of)13). Cytogenic tests include the estimation of mitotic Index assay, according to the formula according to (14).

Results

1-Cytogenic analysis: high mean values of CA and MN (7.90±0.54), (5.00±0.79) respectively was observed in animals inoculated with MMC at (P≤ 0.01) as comparing to animals immunized with CF*LM*Ags with mean values of CA (0.04±0.02) and MN (0.06±0.02), respectivly. WS*LM*Ags-treated MMC with mean values of (0.04±0.02),(0.04±0.02) respectivly, non significant differences (P≤ 0.01) were observed in the mean values of non-immunized animals (0.03±0.00) and (0.03±0.00). The mean values of MI% in animals immunized with WS*LM*Ags were (3.16±0.05) and in WS*LM*Ags+MMC (0.58±0.25), while in animals immunized with CFLMAgs were (4.00±0.45) and in CFLMAgs+MMC (1.52±0.34).All these results were higher than the values in animals treated with MMC (1.08±0.45) and non-immunized non MMC treated (2.03±0.00) animals, (Table-1).

Table -1: percentages (%) of MI, CA and MN in animals immunized with viable-virulent *LM*Ags and treated with MMC.

	Groups	MI%	CA%	MN%
(1)	**MMC.**	1.08 ± 0.45^a	7.90 ± 0.54^a	5.00 ± 0.79^a
(2)	**CFLMAgs.**	4.00 ± 0.45^c	0.04 ± 0.02^c	0.06 ± 0.02^c
	CFLMAgs+ MMC.	1.52 ± 0.34^b	3.8 ± 0.42^b	2.44 ± 0.54^b
(3)	**WSLMAgs.**	3.16 ± 0.05^c	0.04 ± 0.02^c	0.04 ± 0.02^c
	WSLMAgs +MMC.	0.58 ± 0.25^c	0.62 ± 25^c	0.48 ± 0.21^c
(4)	**Negative control.**	2.03 ± 0.00^d	0.03 ± 0.00^d	0.03 ± 0.00^d

(a, b, c, d); different small Letters denoted the significant differences between different groups at (P≤ 0.01).

2:-Detection the concentrations of IFN-y (pg/ml):-The results in table-2 showed that the mean values of IFN-y in MMC treatment was lowest as comparing with animals immunized with CFLMAgs (647.29±194.73) and WSLMAgs (607.29±194.73), the results also showed that the levels of IFN-y in post-challenged with ViLM, and WSLMAgs+MMC was equal to (164.9±4.31) and CFLMAgs+ MMC (271.85±175.2).

Table-2, Levels of IFN-y pg/ml in immunized animals with viable-virulent LMAgs and treated+MMC.

	Concentrations of IFN-y		
	Group	Pre-challenged	Post-challenged
(1)	MMC	0.18 ± 0.08^e	0.159 ± 0.09^e
(2)	CFLMAgs	647.29 ± 194.73^b	560.78 ± 346.24^c
	CFLMAgs MMC		271.85 ± 175.2^d
(3)	WSLMAgs	607.29 ± 194.73^b	184.9 ± 3.31^e
	WSLMAgs MMC		164.9 ± 4.31^e
(4)	ViLM		189.9 ± 0.31^e
(5)	Negative control	100.47 ± 180.33^d	

(a, b, c, d); different small Letters denoted the significant differences between different groups at (P≤ 0.01).

3:-Detection IgG$_1$ pg/ml Antibody titers: The mean values of IgG$_1$ in MMC treated–animals was lowest (0.22±0.06) as comparing with control negative group (12.02±0.06) and animals immunized with CF*LM*Ags (22.23±0.19) and WS*LM*Ags (21.95±1.99) (Table-3). Besides MMC treated-animals expressed lowest values of IgG1 (0.06±0.01) post-challenged with Vi*LM* as comparing with immunized WS*LM*Ags+MMC (15.90±0.76) and CF*LM*Ags+MMC (22.95±1.9), post-challenged with Vi*LM* and control positive group (24.12±0.62).

Table 3, Levels of IgG$_1$ antibody titers pg/ml in immunized animals with variant Lm-Ags and treated with MMC.

	Concentrations of	IgG$_1$ pg/ml	
	Group	**Pre-challenged**	**Post-challenged**
(1)	MMC	0.22 ± 0.06^d	0.06 ± 0.01^d
(2)	CFLMAgs	22.64 ± 0.03^b	25.08 ± 0.02^a
	CFLMAgs+ MMC.		22.95 ± 1.9^b
(3)	WSLMAgs	21.95 ± 1.99^b	22.95 ± 1.99^b
	WSLMAgs+ MMC.		15.90 ± 0.76^c
(4)	ViLM		24.12 ± 0.62^a
(5)	Negative control	12.02 ± 0.06^c	

(a,b,c,d,e) ,different small letters denoted the significant differences between different groups at (P≤ 0.01).

4-Results of cytogenic analysis: The main structural shapes that induced in chromosomes of mice treated with MMC are; ring-shape, centric fusion and fragments, centromeric attenuation, chromatid deletion and micronucleus formation (figure-1&2).
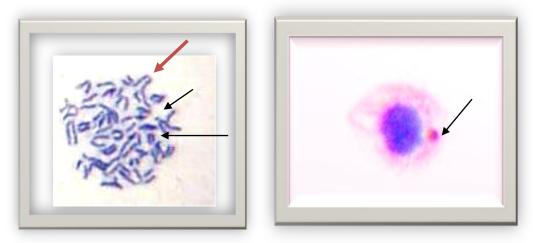
Figure-1:-different shapes of chromosome aberrations in mice treated-MMC; ring shape (long arrow), centromeric attenuation (red arrow) and centric fragment (short arrow).Figure-2:- micronucleus (arrow) in mice treated- MMC (Geimsa stain, 100X).

5-Histopathological examination in control positive group and treated MMC-challenged with Vi*LM*

Histopathological changes were similar but more sever in MMC-treated post-challenged with Vi*LM*. The microscopic examinations of Lungs showed thickening of inter-alveolar septa due to fibrin deposition, infiltration of inflammatory cells and congestion of blood vessels (figure-3). In Liver; acute suppurative inflammation characterized by infiltration of neutophils in Liver parenchyma and lumen of congested B.Vs., as well as granulomatous lesions consisting from aggregation of macrophages in liver parenchyma with dilated sinuses in the animals infected with Vi*LM* (figure-4). Small-multiple granulomatous lesions consisting from aggregation of macrophages and neutophils surrounded by necrotic area in liver parenchyma of animals treated with MMC.The white pulp of spleen showed moderate hyperplasia, congestion of red pulp and proliferation of megakaryocytic cells, mononuclear cells infiltration around congested blood vessels were seen in the kidney.

 6- Immunized CFLMAgs and WSLMAgs-treated MMC challenged with Vi*LM*: The Liver of immunized CF*LM*Ags+Vi*LM* infection showed aggregation of mononuclear cells mainly Lymphocytes around congested blood vessels and in portal area. Small-multiple granulomatous lesions also reported in Liver parenchyma together with proliferation of kupffer cells, while immunized+MMC+infected animals showed large granulomatous lesions consisting from aggregation of neutrophils and mononuclear cells surrounded by large coagulative necrotic area characterized by eosinophilic cytoplasm of hepatic cells with pyknotic or disappeared their nuclei (figure-5). Marked hyperplasia of lymphocytic cells in periarterial sheath and proliferation of mononuclear cells around the sinuses, in white pulp of spleen in immunized-infected animals, while in immunized MMC-infected animals showed moderate hyperplasia of white pulp. The Lungs of immunized animals showed mononuclear cells aggregation around blood vessels, while in immunized MMC+infected animals showed congestion of blood vessels with neutrophils in their lumens together with fibrin network deposition and inflammatory cells infiltration around blood vessels, mononuclear cells aggregation around blood vessels were reported in other examined organs.
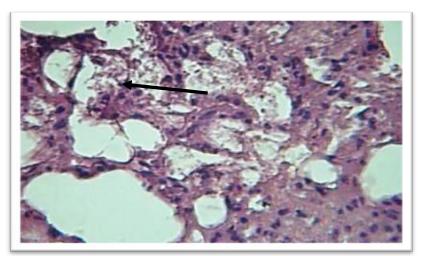
Figure-3:-Fibrin network in Lung alveoli (arrow) of mouse treated MMC, (H&E stain, 40X).
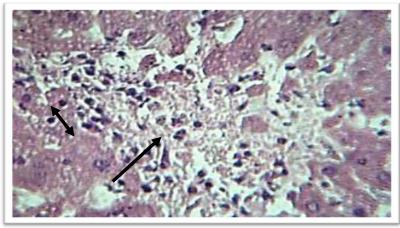

Figure-4:- Liver of mouse treated-MMC and Vi *LM* shows Large area of necrosis (arrow) and small-multiple granulomatous lesions (◄──►) (H&E stain, 40X).
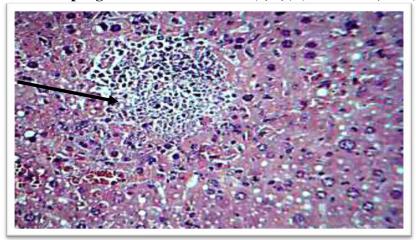

Figure-5: Granulomatous lesions in Liver of mice immunized CF*LM*+MMC challenged with ViLM (➤) (H&E stain, 20X).

Discussion

1-Results of immune responses: The levels of IFN-y and IgG_1 decreased by MMC in animals immunized with *LM* antigens as comparing with non-immunized non-MMC treatment and immunized animals, were varied according to the type of immunizing antigen, may indicated MMC depressant effect on the immune responses, as (15) explained that the chemotherapeutic agents causes depression of immune system, moreover, our results showed

high levels of IFN-y in immunized animals with WS*LM*Ags, may be indicated that immunization with WS*LM*Ags provided good immune responses against opportunistic *LM* infection after treatment with MMC.

2-Cytogenetic results: In this study the concentrations of MMC induced cytotoxicity was equal to 0.4mg/ml. This evidence is in agreement with (5) who recorded that 0.05mg/ml MMC for 24 hrs not sufficient to induced mitotic inhibition and chromosomal aberrations but at concentrations of 2.0mg/ml and above showed complete inhibition of mitotic activity and highly chromosomal aberrations. MMC inhibited mitotic index, increased chromosomal aberrations and micronuclei (16), the present study, reported that MMC induced chromosomal aberrations in mice. However, the main structural chromosome aberration shapes present in our study were; ring-shape, centromeric attenuation, centric fusion and fragments, also micronucleus formation, may be indicated MMC have direct cytotoxic effects on the chromosome chromatid and induce chromosome exchanges, which supported idea mentioned by (17) who reported that different MMC metabolic pathways resulted in ROS generation and some MMC metabolites may form cross-Links to adjacent guanines in DNA. Besides (18) suggested the heterochromatin–specific pairing following MMC treatment reflects the initiation of DNA cross-link repair and formation of exchanges.

In Immunized animals with various *LM*Ags and MMC-treated expressed low levels of chromosomal aberrations and micronucleus as comparing with non-immunized- MMC treated animals and these results are variable according to the type of immunizing antigens, the lowest percentages of CAs and MN reported in animals immunized with CF*LM*Ags and WSLMAgs, may indicated that the strength immune responses diminished the side effects of MMC on the host, therefore, the animals resists bacterial infection. According to these results we suggested the usage immunostimulater agents with MMC as antitumour chemotherapy which is in agreement with (19 and 20).

3-Pathological examination: Suppurative inflammatory reaction predominant in animals infected with *LM* and more extensive in MMC treated+infected animals may be due to exposure to highly virulent *LM* which overcome the innate immune system and disseminated to internal organs to induce tissue damage, These results are in agreement with (21&22), who explained that virulent *LM* disseminated via blood stream to internal organs and induced non-specific inflammatory reaction by Listeriolysin O, with destruction of endothelial cells of blood vessels (23). Multiple granulomatous lesions observed in liver parenchyma of MMC treated- infected animals and large granulomatous lesions in liver of non-treated+infected animals, may indicated the virulence *LM* and strong body defense mechanisms to overwhelm infection (24). These evidences were in consistence with (25&26), who explained that granulomatous reaction indicated strongest body defenses against virulent bacterial infection, moreover, the presence of multiple-small granulomatous lesions in treated+infected animals may be indicated that MMC depressed immune responses by the intensity of granulomatous reactions depend on immune response (27). Mild to moderated pathological lesions observed in internal organs of immunized post-challenged–*LM* and MMC treated+infection, as comparing to control positive group may be due to the stimulation of immune responses which were diminished duo to the side effects of MMC and coincidence with the results of cytogenic analysis and those of immune responses. Lymphoid tissue hyperplasia and mononuclear cells infiltration around blood vessels in internal organs of immunized animals post-challenge with *LM* indicated that *LM*Ags stimulated good immune responses, protected immunized animals against challenge with Vi*LM*. These evidences were agreement with (28 and 29) who reported that lymphoid tissue hyperplasia indicated good immune responses.

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