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Biochemical and pathological studies on the effects of levamisole and chlorambucil on Ehrlich ascites carcinoma-bearing mice

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Summary

Clinicopathological studies on the effects of combining immunostimulant drugs (levamisole) with anti-cancer drugs (chlorambucil) revealed the enhancement of the latter against Ehrlich ascites carcinoma-bearing mice and resulted in a reduction in the size of tumour. An evaluation of liver and kidney functions showed a significant increase of alanine transaminase (ALT), aspartate transaminase (AST) and creatinine in all groups. Histopathological studies of one group that received an intraperitoneal injection of Ehrlich ascites carcinoma cells (2.5×10^6) showed that hepatic parenchyma revealed degenerative changes. The portal area was oedematous and showed rounded cell aggregations. Cell death within hypertrophied Kupper cells was observed in some hepatic cells. The neoplastic emboli could be seen either inside blood vessels or hepatic sinusoids, while another group which had been treated orally with a combination of Leukeran™ (0.2 mg/kg body weight) and levamisole (5 mg/kg body weight) revealed that hepatic parenchyma revealed massive necrosis with proliferative bile duct epithelium. No neoplastic cells were observed without the hepatic parenchyma, while the renal cortex presented a large number of lymphocytes and plasma cells forming bands or aggregates, mainly around the blood vessels. It was concluded that the addition of levamisole to chlorambucil improved the anti-cancer effect of chlorambucil against Ehrlich ascites carcinoma. However, it had adverse effects on the liver and kidneys as shown by liver and kidney function tests and confirmed by histopathology.

Keywords

Carcinoma, Chlorambucil, Control, Ehrlich ascites carcinoma, Levamisole, Mice.

Studi biochimici e patologici sugli effetti di levamisolo e clorambucile su topi portatori di carcinoma ascite di Ehrlich

Riassunto

Gli studi clinicopatologici sugli effetti della combinazione di farmaci immunostimolanti (levamisolo) con farmaci antitumorali (clorambucile) hanno rivelato il potenziamento di questi ultimi nei topi portatori di carcinoma ascite di Ehrlich e la riduzione delle dimensioni tumorali. Una valutazione delle funzioni epatica e renale ha mostrato un aumento significativo di alanina transaminasi (ALT), aspartato transaminasi (AST) e creatinina in tutti i gruppi. Gli studi istopatologici di un gruppo che aveva ricevuto un'iniezione intraperitoneale di cellule carcinoma ascite di Ehrlich ($2{,}5 \times 10^6$) hanno mostrato alterazioni degenerative nel parenchima epatico. L'area portale era edematosa e ha rivelato aggregazioni di cellule arrotondate. Alcune cellule epatiche hanno

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mostrato morte cellulare nelle cellule di Kupfer ipertrofiche. Sono possibili riscontri di emboli neoplastici nei vasi ematici o nei sinusoidi epatici, mentre un altro gruppo trattato oralmente con una combinazione di Leukeran™ (0,2 mg/kg peso corporeo) e levamisolo (5 mg/kg peso corporeo) ha rivelato che il parenchima epatico mostrava una necrosi imponente con proliferazione epiteliale del dotto biliare. Non sono state osservate cellule neoplastiche all'esterno del parenchima epatico, mentre la corteccia renale ha rivelato un numero elevato di linfociti e plasmacellule che formavano bande o aggregati, principalmente intorno ai vasi ematici. La conclusione è che l'aggiunta di levamisolo al clorambucile ha migliorato l'effetto antitumorale di quest'ultimo nel carcinoma ascite di Ehrlich. Tuttavia, ha avuto effetti avversi su fegato e reni come mostrato dai test della funzionalità epatica e renale e confermato dall'istopatologia.

Parole chiave
Carcinoma, Carcinoma ascite di Ehrlich, Clorambucile, Controllo, Levamisolo, Topo.

Introduction

The importance of chemotherapy to cure cancer is increasing, especially with its use as an adjuvant to local therapy. Furthermore, in advanced cases of disease, when the tumour has moved from its place of origin, chemotherapy has an expanding role in efforts to relieve cancer-related symptoms and to prolong life. Despite its shortcomings, chemotherapy, therefore, is an important mode of treatment in oncology and will probably remain so for a considerable time (16). Chlorambucil is an aromatic nitrogen mustard that is useful in the treatment of chronic lymphocytic leukaemia, malignant lymphoma and carcinoma of the ovary (18). Levamisole is an anthelmintic drug that stimulates the parasympathetic and sympathetic ganglia in susceptible worms. It is also an immunomodulator that exerts an immunostimulant action in different animal species when administered at repeated doses of 2.5 mg/kg prior to vaccination. Immunostimulating effects are not well understood. It is believed that an immunomodulator restores the cell-mediated immune function in peripheral T-lymphocytes and phagocytosis by monocytes (15). Furthermore, an immunomodulator appears to stimulate the production of interleukin-2 (IL-2) and lysozyme, thereby enhancing lymphocyte blastogenesis and increasing the level of specific immunoglobulin in the colostrum of vaccinated animals (7, 14). Levamisole, also used as an immunostimulant in human cancer therapy, is a strong inhibitor of tumour aerobic glycolysis. It diminishes growth of Ehrlich ascites carcinoma (12). Ehrlich mouse ascites tumour became one of the widely used experimental cancer cells grown in the peritoneal cavity of Swiss albino mice. After inoculation, ascites became apparent in most mice within a few days and exhibited marked ascites within 10-12 days (17).

The aim of our study was to examine some biochemical and pathological changes after using anticancer drugs, chlorambucil or levamisole and a combination of the two, in mice affected by Ehrlich ascites carcinoma.

Materials and methods

Experimental animals

A total of 100 adult female Swiss albino mice (average 18-20 g in weight) were obtained from the laboratory animal farm of Veterinary Medicine at Zagazig University in Egypt. All mice were reared under strict standard hygienic measures and were fed on a balanced ration with a good source of water *ad libitum*.

Ehrlich ascites carcinoma cells

The parent line of Ehrlich ascites carcinoma cells was kindly supplied by the National Cancer Institute of Cairo University. The tumour line was maintained by serial intraperitoneal transplantation of Ehrlich ascites carcinoma 2.5×10^6 tumour cells/0.2 ml in female Swiss albino mice.

Anti-neoplastic drugs

The following drugs were used:

- Leukeran™ chlorambucil tablets BP 2 mg (Heumann Pharma GmbH for Glaxo Wellcome GmbH & Co., Bad Oldesloe)

- immunostimulant agent (levamisole 10%) levamisole hydrochloride (PharmaSewede, Egypt).

Experimental design

Female Swiss mice were divided randomly into five groups (20 mice per group). Group 1 was kept as the control group, Group 2 received intraperitoneal injection of by 2.5×10^6 Ehrlich ascites carcinoma cells, Group 3 was treated orally with Leukeran™ 0.2 mg/kg body weight), Group 4 was treated orally with levamisole (5 mg/kg body weight) and Group 5 was treated orally with a combination of Leukeran™ and levamisole each day, using a bent stainless steel stomach tube (Table I).

Blood sampling

Ten mice in each group were used for blood collection from the retro-orbital venous plexus. Blood samples were taken without anti-coagulant in a sterile test tube for separation of serum which was used to measure biochemical parameters. Blood samples were collected at 12 days post intraperitoneal inoculation of Ehrlich ascites carcinoma cells.

Clinicopathological studies

The serum total protein and serum albumin levels were measured (5, 6). The serum globulin level was calculated by subtracting the albumin obtained from the total protein obtained. The serum aminotransferase activities of aspartate (AST) and alanine aminotransferase (ALT) were determined colorimetrically (19). The serum creatinine level was also determined colorimetrically (13).

Histopathology

Specimens from the peritoneum, liver, kidneys and spleen were fixed in 10% neutral buffered formalin paraffin; sections of 5 µ thickness were prepared from all specimens and were stained by haematoxylin and eosin (H&E) and examined microscopically.

Statistical analysis

The data obtained from this investigation were statistically analysed using the Student *t* test according to Tamhane and Dunlop (21).

Results and discussion

This experiment was designed to illustrate the effect of the immunostimulant drug on the efficiency of the anti-tumour drug. Clinical signs observed were distended abdomen, the highest body weight was found in Groups 2, 3 and 4 (Fig. 1) as a result of tumour growth which creates ascetic fluid rich in free neoplastic cells (2) and the lowest weight was observed in Group 5 (Table II). On the other hand, the highest survival rate was recorded in Group 5. This may be due to the fact that levamisole inhibits the growth of a transplanted human tumour in Swiss mice. The use of mice as tumour hosts enabled discrimination between the angio-genesis inhibitory effect of levamisole and its assumed immuno-stimulatory effect (8). This could be

Table I
Experimental design of groups, number of mice in each group, type of treatment, daily administration dose and time of sampling

Group	No. of mice	Treatment	Ehrlich ascites carcinoma	Dose	Route	Duration	Time of sampling
1	20	Normal control	–	–	–	12 days	Day 12
2	20	Cancer-bearing mice	2.5×10^6 EAC	–	Intrapertoneal	12 days	Day 12
3	20	Chlorambucil	2.5×10^6 EAC	0.2 mg/kg body weight	Oral	12 days	Day 12
4	20	Levamisole	2.5×10^6 EAC	5 mg/kg body weight	Oral	12 days	Day 12
5	20	Chlorambucil and levamisole	2.5×10^6 EAC	0.2 + 5 mg/kg body weight	Oral	12 days	Day 12

EAC Ehrlich ascites carcinoma

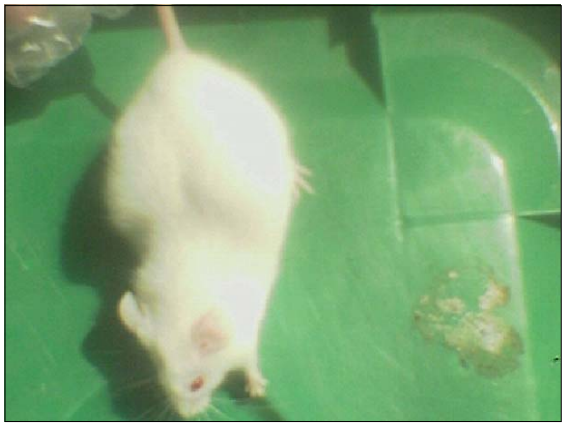

Figure 1
Ehrlich-bearing mice showing ascites (Group 2)

due to chlorambucil producing its anti-tumour effect by inducing apoptosis-associated membrane changes that result in rapid clearance of the apoptotic cells by the immune system (3).

Regarding the results of total proteins, albumins and globulin levels showed a significant decrease in total proteins and albumin levels in Group 2 (Table III). This may be attributed to increased mitotic division of neoplastic cells with high bloody fluid withdrawal and capillary permeability which enable the escape of plasma proteins into the peritoneal cavity and may also be due to hepatic cell necrosis (9). In addition, total proteins may decrease in animals with liver disease (4). Hypoalbuminaemia in domestic animals may be due to excessive nephritis, certain cases of massive ascites and can also be associated with liver disease (4). These results confirmed the body weight result which showed a significant increase in Group 2 as a result of tumour growth which formed ascetic fluid that was rich in free neoplastic cells (2). Total protein and albumin levels increased in Group 5 towards a normal level but were still low, possibly due to the anti-tumour effect of chlorambucil by inducing apoptosis-associated membrane changes that result in the rapid clearance of the apoptotic cells by the immune system (3) and helped to reduce the growth of Ehrlich ascites carcinoma cells by levamisole which decreased ascites in mice (12). Serum globulin decreased in Group 3 which could have been due to the immunosuppressive effect of chlorambucil (20).

In regard to the liver and kidney function, evaluations revealed a significant increase of ALT, AST and creatinine in all groups (Table III). This could be attributed to the presence of hepatic and renal damage as a result of cancer cell invasions (11). Moreover, Tofani *et al.* (22) indicated that large tumour masses and the associated long-lasting necrosis are considered to cause metabolic overloading of the liver while chlorambucil creates hepatic toxicity which leads to an increase of ALT and AST (23). Finally, levamisole treatment induced acute hepatic degeneration and necrosis (10), hydropic degeneration of

Table II
Effect of Ehrlich ascites carcinoma and administration of drugs on body weight and survival percentage in mice
(mean values ± standard error)

Group	Parameters	Body weight	No. of mice	No. of mice that survived	Survival rate
1	Controls	22.54 ± 0.59	20	19	95%
2	Cancer-bearing mice	28.45 ± 0.74*	20	11	55%
	Difference (%)	+26.22			
3	Chlorambucil	26.14 ± 0.66*	20	16	80%
	Difference (%)	+15.97			
4	Levamisole	25.00 ± 0.68*	20	12	60%
	Difference (%)	+10.91			
5	Chlorambucil + levamisole	23.82 ± 0.66 NS	20	17	85%
	Difference (%)	+5.67			

* significant difference at $p \leq 0.05$
NS not significant

Table III
Effect Ehrlich ascites carcinoma and administration of chlorambucil and levamisole on some biochemical investigations in mice

Group	Parameters	Total proteins (g/dl)	Albumin (g/dl)	Globulins (g/dl)	ALT (units/l)	AST (units/l)	Creatinine (mg/dl)
1	Control	7.28 ± 0.14	3.07 ± 0.05	4.21 ± 0.15	18.36 ± 0.61	37.05 ± 0.02	0.63 ± 0.04
2	Cancer-bearing mice	5.60 ± 0.17*	1.52 ± 0.23*	4.07 ± 0.11 NS	35.78 ± 0.02*	78.12 ± 0.06*	1.36 ± 0.04*
	Difference (%)	–23.07	–50.48	–3.32	+94.88	+110	+115.87
3	Chlorambucil	4.51 ± 0.33*	2.18 ± 0.07*	2.32 ± 0.31*	44.84 ± 0.03*	87.72 ± 0.04*	1.65 ± 0.08*
	Difference (%)	–38.04	–28.99	–44.89	+144.22	+136.76	+161.90
4	Levamisole	5.54 ± 0.17*	2.78 ± 0.03**	2.76 ± 0.14*	47.30 ± 0.02*	100.24 ± 0.01*	1.91 ± 0.06*
	Difference (%)	–23.90	–9.44	–36.57	+157.62	+170.55	+203.17
5	Chlorambucil + levamisole	6.91 ± 0.33 NS	2.76 ± 0.10**	4.15 ± 0.42 NS	56.64 ± 0.80*	119.82 ± 0.03*	1.89 ± 0.12*
	Difference (%)	–5.08	–10.09	–1.66	+208.49	+223.40	+200

ALT alanine transaminase
AST aspartate transaminase
NS not significant
* significant difference at $p \leq 0.05$
** highly significant difference at $p \leq 0.01$

hepatocytes, congestion of the renal blood vessels and degenerative changes in the epithelial lining of renal tubules (1), which were confirmed by histopathological studies (Figs 2, 3, 4, 5 and 6).

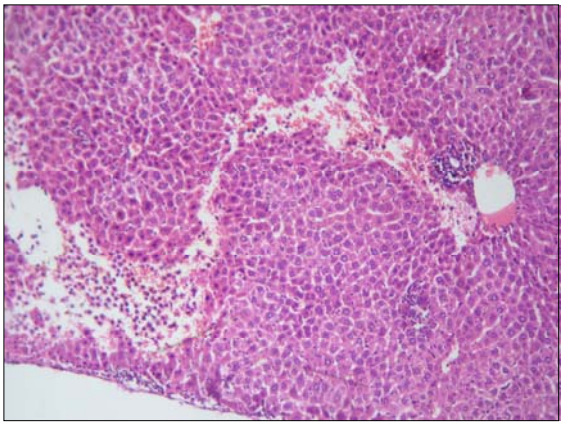

Figure 2
Mice liver showing isolated tumour cells and haemorrhage within attract in the hepatic parenchyma
(Group 2)
(H&E, ×120)

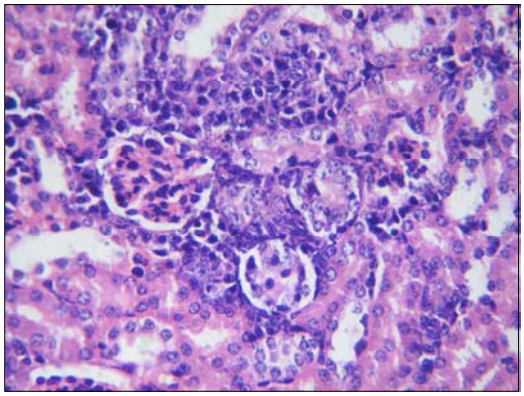

Figure 3
Mice kidney showing interstitial nephritis
(Group 3)
(H&E, ×300)

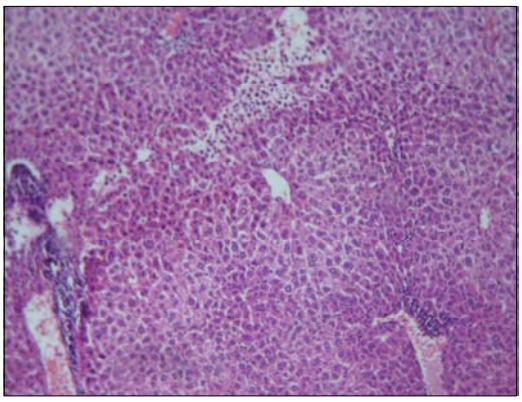

Figure 4
Mice liver showing little neoplastic cells, haemorrhaging and massive necrosis in the adjacent parenchyma
(Group 3)
(H&E, ×120)

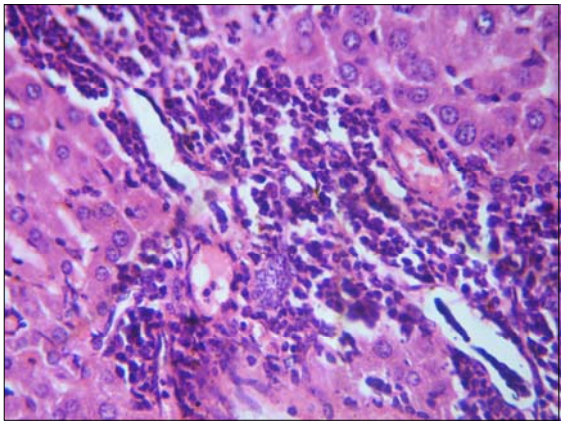

Figure 5
Mice liver showing portal lymphocytic and granulocytic aggregations and hepatic necrosis
(Group 4)
(H&E, ×300)

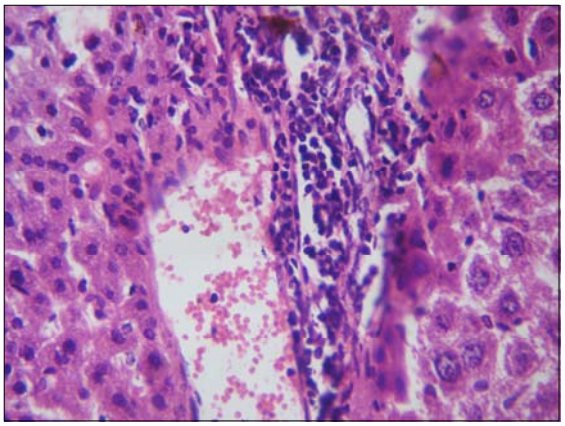

Figure 6
Mice liver showing hepatic necrosis and portal round cell infiltration
(Group 5)
(H&E, ×300)

Conclusions

The following conclusions were drawn from our study:

- levamisole can be used as a anticancer agent through its immunostimulant effect
- the combination of levamisole and chlorambucil improved the anti-cancer effect of the latter against Ehrlich ascites carcinoma which increased apoptosis of Ehrlich ascites carcinoma and the survival rate of the mice with cancer, but it had adverse effects on the liver and kidneys as revealed by liver and kidney functions test and confirmed by histopathology.

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